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Propagation of Measles Virus in Human Carcinoma Cells. (22643)

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(Introduced by J. F. Enders)
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Because they could not regularly be reproduced, early claims of the propagation of measles virus in tissue cultures have not met with wide acceptance. However, Enders and Peebles(1) reported that an infectious agent could be isolated from the throat or blood of measles patients and readily grown in cultures of human kidney cells.

It seemed desirable to find other cells in which the virus may be propagated since human kidneys are not readily available and monkey kidney cells, although susceptible, frequently carry a latent agent which may cause lesions similar to those of measles virus. To this end, Dr. George Foley put at our disposal the KB strain of human epidermoid carcinoma cells, originally isolated by Dr. Harry Eagle(2). These cells grow profusely in a relatively simple and well defined medium containing 10% horse serum(3,4).

In tubes to which a suspension of these cells were added, confluent sheets of growth were seen after 2 or 3 days. At this time, the cells were inoculated with the fluid from the 23rd passage in human kidney cell cultures of a strain (Edmonston) of measles virus(1).

Daily microscopic examination of the cultures revealed on the 4th or 5th day, 6 to 10 areas of "giant cell" or syncytial formation comparable to those described by Enders and Peebles(1) in cultures of human renal cells. By the following day, however, these "lesions" had fallen off the glass and the clear area was quickly overgrown by cells of normal appearance. Two to 3 days later, (Fig. 1), a second but larger group of giant cells appeared both in the same areas and elsewhere throughout the cell sheet. This process of simultaneous cell destruction and multiplication continued until complete destruction of the culture by the virus at the end of 5 weeks.

During this period, culture fluid from the fast growing yet infected primary culture was used repeatedly to start subcultures in which the same changes were observed and in which the cells underwent the same fate as those of the primary culture within about the same period. Only in the final stages were acidophilic inclusion bodies seen in several of the cells that were comparable to those described as occurring abundantly in normal human and monkey renal cells(1). Further evidence that these lesions were caused by measles virus was afforded by the specific fixation of complement using as antigens fluids from infected cultures in the presence of measles convalescent serum. Only those fluids, however, taken from cultures that were inoculated with the virus 3 to 4 weeks previously were shown to fix complement under these conditions.

From these observations it is concluded that the measles virus may be propagated in

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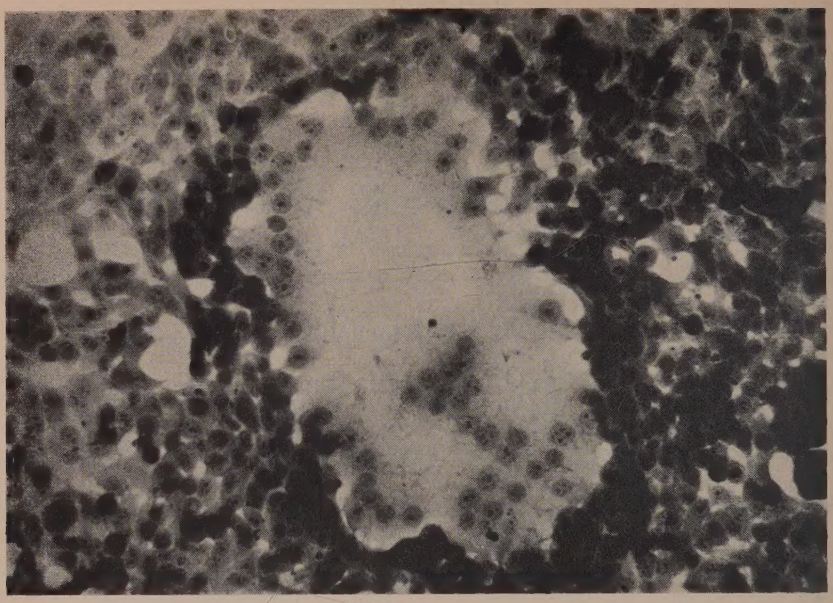

FIG. 1.

the KB strain of human carcinoma cells.

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Lipid Metabolism in Kidney Undergoing Compensatory Hypertrophy.* (22644)

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Previous studies involving "regenerating" liver in rats(1), and the prostate and seminal vesicle tissue of non-castrated and castrated rats with and without testosterone treatment (2), suggested that there was an increased P^{32} uptake in all of the Schmidt-Thannhauser (3) fractions that was associated with cellular hypertrophy. Neither of these conditions can be considered as a hypertrophy, pure and simple, however, since the hypertrophy seen in the first 24 hours in regenerating liver is, in a sense, a preparative state for an ensuing cellular hyperplasia in that it occurs prior to the appearance of mitosis, while that in the prostates and seminal vesicles occurred in the

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† Taken in part from a dissertation submitted by Elinor Levin for the partial fulfillment of the degree of Doctor of Philosophy in Physiological Chemistry from Wayne University Graduate School.

presence of actively dividing cells. It was desirable to study the phosphorus metabolism associated with a pure hypertrophy in order to determine whether the changes observed to occur during cell growth were related to or were independent of mitosis.

When one kidney of an experimental animal is surgically removed, the remaining kidney is observed to undergo compensatory hypertrophy(4,5). In this case the complicating factors mentioned above appear to be absent or greatly minimized, and thus this tissue presents itself as one which might profitably be studied.

Experimental. Six male Fisher strain rats, 3-4 months old, were used in each experimental group. The animals were maintained on a stock diet of Purina laboratory chow and water *ad libitum* before and after unilateral nephrectomy. Nephrectomy was accomplished by making a lateral incision about ¾ inches long on the left flank, just below the inferior border of the rib cage. The kidney was raised from the incision and the adrenal gland stripped away and pushed back into the cavity. The pedicle of the kidney was ligated, the kidney excised, and the incision closed in 2 layers.

Preliminary experiments(6) were carried out to determine the percent increase of the intact kidney weight over that of the excised kidney at various times following nephrectomy. The results, calculated on both a moist and dry basis, showed that the greatest increase in weight of the intact kidney occurred about the third day, and rate of increase leveled off at about the seventh day. Therefore, the animals were sacrificed either 3 or 7 days following nephrectomy. Kidneys taken from the right side of non-nephrectomized animals served as controls.

Four hours after giving each rat a subcutaneous injection of 0.45 μc of radioactive phosphorus‡ per g body weight the animal was sacrificed. The abdominal cavity was exposed, and the intact kidney was perfused with ice cold physiological saline *in situ* via the inferior vena cava. The kidney was removed, cut into small pieces, and homogenized in 10% trichloroacetic acid containing 0.4 M $MgCl_2$(7). The phosphorus-containing fractions shown in Table I were then prepared by methods previously described(8,9, 10).

One kidney did not provide enough tissue for the fractionation described above and also for unsaturated fatty acid determinations. Therefore, additional groups of control and experimental animals were prepared in a manner identical to that described, and dienoic, trienoic, tetraenoic, and pentaenoic acids were determined on the perfused kidneys following their homogenization in physiological saline (11).

Results. Three days after nephrectomy the intact kidneys weighed an average of 10% more than those removed; after 7 days they weighed 23% more, and after 20 days they were 27% heavier than the controls. Increases in the amount of cephalin and lecithin in the hypertrophying kidneys were usually apparent 7 days after nephrectomy. However, there was a decreased P^{32} uptake by these 2 phospholipids 3 days after nephrectomy, that had returned to the normal (control) levels by 7 days (Table I). No changes in either amounts of phosphorus or of P^{32} uptake by any of the remaining fractions studied were observed either 3 or 7 days after unilateral nephrectomy (Table I). These results, especially those concerning P^{32} uptake by the phospholipid and acid-soluble fractions, were in contrast to those seen in the experiments involving hypertrophying liver 24 hours after partial hepatectomy(1) and seminal vesicles and prostates of castrated rats following testosterone treatment(2).

Samples of all tissues used in the study were fixed in Bouin's solution and prepared for histological examination(1). The mitotic activity of the kidney 3 and 7 days after nephrectomy was no greater than that seen in organs taken from the unoperated controls. While not enough is known to postulate that there are biochemically distinct types of hypertrophy, it seems possible tentatively to hypothesize as follows: If a tissue is subjected to a stimulus capable of eliciting cellu-

‡ The radioactive phosphorus used in these experiments was furnished by the Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE I. P^{32} Uptake and Concentration of Phosphorus in Rat Kidney Undergoing Compensatory Hypertrophy.

Fractions	Controls		3-day nephrectomy		7-day nephrectomy	
	P^{32} uptake*	μg P/mg N	P^{32} uptake	μg P/mg N	P^{32} uptake	μg P/mg N
Acid-soluble	1706 (79)†	26.7 (1.3)	1604 (70)	25.7 (.8)	1776 (58)	30.2 (.9)
Total phospholipids	488 (18)	29.5 (.8)	501 (15)	30.3 (1.0)	519 (23)	33.1 (.4)
Cephalin	333 (14)	14.1 (.4)	284 (10)	12.5 (.2)	346 (20)	16.1 (.3)
Lecithin	809 (52)	11.5 (.4)	780 (21)	13.0 (.4)	877 (90)	12.9 (.3)
Sphingomyelin	93 (4)	3.6 (.1)	87 (3)	3.5 (.2)	99 (4)	3.6 (.1)

* Figures for P^{32} uptake are concentration coefficients calculated as follows:

$$\frac{\text{Counts/min. in fraction}/\mu\text{g phosphorus in fraction}}{\text{Counts/min. injected}/\mu\text{g of body wt}}$$

† Figures within parentheses are stand. errors of mean values.

lar growth (hypertrophy) that is accompanied or followed closely by cellular increase (hyperplasia), it may be expected to show an increased P^{32} uptake in the fractions isolated by the Schmidt-Thannhauser procedure. If, however, the stimulus elicits cell hypertrophy only, it need not produce a change in the rate of uptake of P^{32} in these fractions.

The changes in the concentrations of the unsaturated fatty acids were greater in the presence of compensatory hypertrophy of kidneys, seen in the present experiments, than were observed in the case of liver hypertrophy following partial hepatectomy(12). The dienoic and tetraenoic acid contents were increased, and the trienoic and pentaenoic acids decreased 3 days after nephrectomy (Table II). By the seventh day the amounts had essentially returned to normal. The changes observed then, seemed to be associated with a cell that was rapidly enlarging in size at 3 days, rather than with the enlarged cell itself (at seven days). It is of interest, and perhaps significant, that the compensatory response in the intact kidney 3 days after nephrectomy, whatever its nature, was associated with an increase in only those fatty acids essential for growth, *i.e.*, dienoic and tetraenoic acids, and a decrease in trienoic and pentaenoic acids.

TABLE II. Concentration of Unsaturated Fatty Acids in Rat Kidneys Undergoing Compensatory Hypertrophy.*

	Control (4 animals)	3-day nephrectomy (6 animals)	7-day nephrectomy (4 animals)
Dienoic	94 (4)	112 (6)	86 (2)
Trienoic	16 (4)	4 (2)	9 (2)
Tetraenoic	123 (2)	137 (2)	109 (2)
Pentaenoic	22 (3)	14 (1)	16 (1)

* μg of unsaturated fatty acid/mg nitrogen. Figures in parentheses are stand. errors of the mean values.

Summary. The increased P^{32} uptake of the various phosphorus-containing fractions during hypertrophy in liver and prostatic tissue is not exhibited during renal hypertrophy. It is suggested that the P^{32} increases seen in the liver and prostatic tissue during hypertrophy are associated with subsequent mitotic activity found in these tissues.

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Conversion of C^{14}-Arterenol to Epinephrine *in vivo*.* (22645)

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Considerable progress has been made in elucidating the pathway of epinephrine biosynthesis by tracer studies. It has been demonstrated that phenylalanine and tyrosine are precursors of epinephrine *in vivo*(1,2) and *in vitro*(3). Adrenal epinephrine has been obtained after injection of C^{14}-3,4-dihydroxyphenylalanine and C^{14}-3,4-dihydroxyphenethylamine into rats(4). Injection into rats of C^{14}-methionine labelled in the methyl group produced C^{14}-epinephrine in the adrenal gland(5). Although indirect evidence has been offered(6,7,8), the main evidence for the conversion of arterenol to epinephrine is based on the experiments of Bülbring(9), who used bioassays to measure the increase of epinephrine in adrenal preparations incubated with arterenol and methyl donor *in vitro*. Several attempts to repeat Bülbring's experiments in this laboratory gave negative results. The present paper reports a search for C^{14}-epinephrine in the adrenals of rats after the intraperitoneal injection of C^{14}-arterenol.

Materials and methods. The α-C^{14}-dl-arterenol hydrochloride was synthesized by Howton, Mead, and Clark(10). Its activity was 16 mc per millimole. The sample had deteriorated somewhat on storage and in some experiments was purified just before use by ion exchange chromatography. A solution of 8 mg of the impure C^{14}-arterenol in 8 ml water was passed onto a column (24 cm long, 2.4 cm O.D.) of Dowex 50 resin (200-400 mesh, 8% crosslinked) in the hydrogen form in 0.5 *N* HCl. The column was developed with 0.5 *N* HCl, and fractions of approximately 20 ml were collected. The arterenol fractions (#83-107) were combined, passed through a column of 250 g Dowex 1 resin in the acetate form (1.5 times the theoretical amount) to remove the chloride ions, and lyophilized. Its purity was determined by chromatography on Whatman #1 paper using as developing solvent phenol-0.1 *N* HCl (85:15 (wt./vol.)) saturated with SO_2. The chromatogram was cut into segments and the radioactivity measured in a windowless flow counter (Fig. 1).

Four adult male Long-Evans rats were injected intraperitoneally with α-C^{14}-dl-arterenol hydrochloride twice a day for 3 days and sacrificed under nembutal anesthesia 24 hours after the last injection (Exp. 1 and 2; 2 rats per experiment). Six other rats were given 6 injections at hourly intervals and sacrificed one hour after the last injection (Exp. 3, 4 and 5; 2 rats per experiment). The adrenal glands were removed and kept frozen until used. Adrenals of 2 rats were ground in a glass homogenizer with 2 *N* acetic acid. This suspension was divided into 2 equal parts. One part was used for paper chromatography. It was evaporated *in vacuo* at 45°-50° C., and the residue extracted with several portions of ethanol (total volume, 1.0 ml.) and centrifuged. The supernatant was evaporated to less than 0.5 ml with a stream of nitrogen and the entire solution spotted on Whatman #1 paper. The chromatogram was developed overnight in the phenol solvent, washed with benzene, dried, sprayed with $K_3Fe(CN)_6$ solution(11), and counted as above.

The other half of the suspension was used for the determination of specific activity after clarifying by adjusting to pH 6.5 with 2 *N* NH_4OH and centrifuging. An aliquot of the supernatant was assayed for epinephrine by a modified Weil-Malherbe procedure‡(12). To the rest of the extract 15.0 mg of unlabelled l-epinephrine (as 27.3 mg of the syn-

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† Research Fellow of the Los Angeles County Heart Assn., 1955-56.

‡ We wish to thank Dr. Samuel Eiduson for the epinephrine determinations.

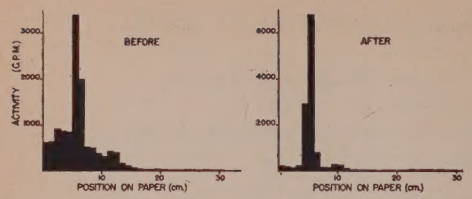

FIG. 1. Distribution of radioactivity on paper chromatogram of α-C^{14}-dl-arterenol before and after ion exchange resin treatment.

thetic bitartrate) was added as carrier. The l-epinephrine was crystallized by adjusting the solution to pH 8.8 with 2 *N* NH_4OH. The precipitate was filtered with suction, washed 3 times with cold water and once with ethanol, plated out, and counted. It was found impossible to separate epinephrine from contaminating radioactive arterenol in control experiments by a few crystallizations (also reported by Udenfriend and Wyngaarden(4)), so a method was devised which removed arterenol by virtue of its reactivity with pyridoxal-5-phosphate(13) (Table I). Sodium pyridoxal-

TABLE I. Separation of Labelled Arterenol from Carrier Epinephrine in Control Experiments.

		c.p.m./mg	% arterenol in epinephrine
A. Original mixture		8100	.40
Crystallized	1×	6800	.34
"	2×	5500	.27
"	3×	4800	.24
Pyridoxal-5-phosphate	1×	325	.016
B. Original mixture		67	.033
Crystallized	1×	42	.021
Pyridoxal-5-phosphate	1×	3.4	.0017
"	2×	1.1	.0006

5-phosphate solution was prepared from 1 mg of the calcium salt§ (approximately 60% pure) in 0.1 ml of 0.5 *M* phosphate buffer at pH 6.8. The epinephrine sample, which had been redissolved in acetic acid and adjusted to pH 6.8, was allowed to react with the sodium pyridoxal-5-phosphate solution for 30 minutes at 45°C. Then the epinephrine was crystallized, filtered, washed, plated, and counted as before. A second incubation and crystallization was usually sufficient to obtain constant activity.

§ Obtained from the Merck Institute for Therapeutic Research, Rahway, N. J.

TABLE II. Conversion of C^{14}-Arterenol to Epinephrine.

Exp. No.	Hr	Total counts injected	Adrenal epinephrine + carrier (total c.p.m.)	Specific activity of adrenal epinephrine (c.p.m./μM)
1	72	6×10^7	683	
2	72†	6×10^7	955	
3	6	2×10^7	239	4,994
4*	6	2×10^7	383	23,728
5*	6	2×10^7	285	11,463

* Arterenol purified by ion exchange resin treatment.

† Marsilid, 100 mg/kg inj. daily.

Results. It can be seen from Table II that radioactive arterenol injected into rats was converted to adrenal epinephrine. Significant labelling of epinephrine occurred even during the 6-hour experiments. The chromatographic evidence for the presence of radioactive epinephrine is illustrated in Fig. 2, which shows the distribution of radioactivity in a typical paper chromatogram of the adrenal extract (Exp. 5). The chromatogram had been sprayed before counting and the positions of highest activity were found to correspond exactly with the colored spots due to endogenous amines.

As described by Howton, *et al.*(10), a contaminant in the radioactive arterenol has an R_F corresponding to 3, 4-dihydroxyphenethylamine. The possibility that this contaminant may have been responsible for the radioactive epinephrine in the adrenal gland does not appear to be likely, since the specific activities of adrenal epinephrine in Exp. 4 and

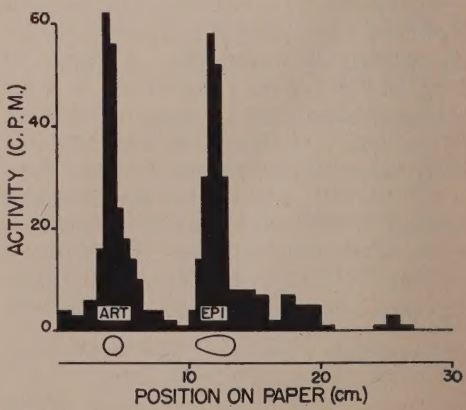

FIG. 2. Distribution of radioactivity on paper chromatogram of adrenal extract from rat inj. intraper. with α-C^{14}-dl-arterenol.

5 are higher than in Exp. 3 even though the contaminant was almost completely removed in the purification of the arterenol.‖

These experiments represent the first direct evidence that arterenol is converted to epinephrine and completes the reaction sequence postulated to lead from phenylalanine to epinephrine.

Summary. C^{14}-epinephrine has been found in the adrenal glands of rats administered a-C^{14}-arterenol intraperitoneally. Its presence has been demonstrated by paper chromatography and by isotope dilution using a new method, involving reaction with pyridoxal-5-phosphate, for separating these catecholamines.

‖ We wish to thank Dr. William Drell who developed this method and demonstrated that it completely separated arterenol from epinephrine and 3,4-dihydroxyphenethylamine. Furthermore, a paper chromatogram of carrier 3,4-dihydroxyphenethylamine hydrochloride which had been recrystallized from a solution containing the purified C^{14}-arterenol hydrochloride showed no radioactivity at the R_F of the carrier.

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Investigations on Polyvinylpyrrolidone in Mice Given Brucella Endotoxin. (22646)

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Studies in these laboratories with endotoxins prepared from various species of Gram-negative bacteria, including brucellae, have confirmed the observations that these endotoxins cause profound vascular disturbances in animals, terminating in collapse and death (1,2). More precise measurements with non-lethal doses of brucella endotoxin have shown that the daily temperature rhythm of mice is affected by small amounts of the endotoxin (3). The lethal effects of endotoxin in mice can be prevented by administration of adrenal corticosteroids or chlorpromazine, and tolerance to lethal doses of endotoxin can be produced by administration of sublethal amounts of heterologous endotoxins(1,4,5,6). The hypothermia of mice given brucella endotoxin also can be counteracted with added corticosteroid(7).

The present studies were undertaken to determine if the polymer, polyvinylpyrrolidone (PVP), would protect mice against brucella endotoxin. Originally introduced as a plasma expander by Hecht and Weese(8), interest turned to using PVP as a "detoxifying" agent. Toxic substances not usually excreted in the urine were reported to be "adsorbed" to PVP and excreted as such(9,10,11). PVP not only protected animals, but it was described as also effective in the treatment of human cases of tetanus and of diphtheria(12,13,14). According to these investigators, the most effective form of PVP was incorporated in the preparation known as Periston N (PVPN), having a smaller molecular weight than the original PVP (molecular weights: PVP~100,000, PVPN~12,600). More recently in a therapeutic trial on 10 patients with severe tetanus, no benefit attributable to PVPN was noted(15). Likewise, in mice PVPN given 3 hours after tetanus toxin was ineffective, but it protected when given within one hour after

TABLE I. Statistical Analysis of Data Presented in Fig. 1.

Comparison No.	Mortality rates compared: Stock	Time, hr	PVPN*	X^2	P
1	ABC	24	0	13.73	<.01
	ABC	24	+		
2	ABC	48	0	27.53	<.01
	ABC	48	+		
3	DBC	24	0	.06	>.32
	DBC	24	+		
4	DBC	48	0	.42	>.32
	DBC	48	+		
5	ABC	24	0	3.09	≃.08
	DBC	24	0		
6	ABC	48	0	1.34	>.16
	DBC	48	0		
7	ABC	24	+	25.49	<.01
	DBC	24	+		
8	ABC	48	+	31.02	<.01
	DBC	48	+		

* 0 = No PVPN inj. + = PVPN inj.

toxin administration(14). In the present investigations PVPN* was tested for a possible protective effect against brucella endotoxin, the 2 agents being administered concomitantly.

Materials and methods. Mice, 5 to 6 weeks of age, were used for testing. The mice had Purina Fox Chow and tap water *ad libitum* and were kept in an airconditioned room, maintained at about 78°F, illuminated by day and darkened by night. A first series of experiments was carried out on hybrids of the A and C stocks (ABC mice). Because of availability, further experiments were carried out on DBC mice, obtained by mating DIF_1 hybrids to the D (dilute brown) stock. As the results on DBC mice were found to be at variance with those previously obtained on ABC mice, a third series of experiments was undertaken. The effects of endotoxin in each of these additional experiments with and without added PVPN were compared alternately in ABC and DBC mice. A Boivin-type of endotoxin made from a smooth culture of *Brucella melitensis* was employed, the details for its preparation having been presented elsewhere(3). Different lots of endotoxin were employed, the doses averaging 1.5 mg per 20 g of body weight. But in any given experiment the same lot of endotoxin was used. A 6% solution of PVPN was used in a dose of 0.5 ml per mouse. All injections were made into the tail vein. Except for one experiment on ABC mice in which it was given 5 minutes after the endotoxin, the PVPN was introduced simultaneously through the same syringe containing the endotoxin. Mortality rates are reported for 24 and 48 hours post-injection. The rates at these times were representative of the PVPN effect recorded in some experiments at shorter and longer intervals as well.

Results. The results of all the experiments are summarized in Fig. 1, and a statistical analysis of these data is shown in Table I. As noted in Fig. 1, a total of 453 ABC mice and 350 DBC mice were used in these studies. The outstanding and consistent result was that ABC mice given endotoxin and PVPN survived in significantly larger numbers than DBC animals treated in the same manner. While PVPN protected ABC mice against the lethal action of the endotoxin, this protection

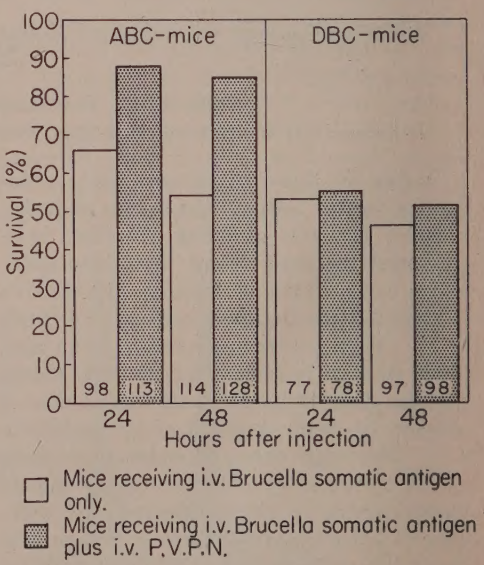

FIG. 1. Comparative survival rates in populations of ABC mice and DBC mice given brucella endotoxin alone, and brucella endotoxin plus Periston N (PVPN). No. of mice constituting each treatment group is shown at bottom of corresponding column.

* Obtained from Die Farbenwerke Bayer, Leverkusen, Germany.

was lacking in the DBC mice, (Fig. 1) from the rates recorded at 24 as well as at 48 hours after injection of endotoxin. When the number of mice comprising these comparative studies is taken into consideration, the differences in per cent of survivals are quite significant. That PVPN protected ABC mice against endotoxin is supported by the results of the statistical analyses given in comparisons 1 and 2 of Table I.

The data in Fig. 1 also suggest that ABC mice may be less susceptible to the endotoxin than the DBC mice, and this might account for the protection offered to ABC mice by PVPN. The differences in survival rates between ABC and DBC mice at 24 and 48 hours after injection are however slight, and they are not significant at the 5% level, as seen in comparisons 5 and 6 in Table I. This slight difference in the susceptibility to the endotoxin does not appear to be sufficient to explain the large difference between ABC and DBC in their response to PVPN. The latter difference is analyzed in comparisons 7 and 8 in Table I, based on survival rates in those groups of ABC and DBC given PVPN as well as endotoxin. Whether data for survival rates at 24 or 48 hours are analyzed, the inter-population differences in survival rates are significant below 1% ($P<0.0005$).

Discussion. These experiments demonstrate that PVPN provides protection for mice against Brucella endotoxin. PVPN significantly increased survival rates of ABC mice when given 5 minutes after endotoxin injection, as well as when given in the same syringe with the endotoxin. But this protection is limited, with populations of ABC mice protected, but not DBC mice. Only a single injection of PVPN was given in evaluating the effect on survival rates. It would be of interest to see whether repeated injections of PVPN may have more general protective effects. In evaluating the effects of polymers in animals given endotoxin, two major considerations must be kept in mind. There exist not only intra-species differences in the response to the polymer, as the present experiments emphasize, but varying results must be anticipated when interpreting the differences between species of animals. When PVP alone is administered to dogs, shock is produced(16). Further differences within the same species may be forthcoming if there is a variation in the molecular weight of the polymer. Moreover, under certain experimental circumstances, related compounds of larger molecular weight have been found to be harmful in rabbits(17).

Summary and conclusions. Brucella endotoxin was administered intravenously to groups of mice and survival rates were recorded at 24 and 48 hours post-injection. The effect of a synthetic polymer on such survival rates was evaluated in 2 genetically different populations of mice—ABC mice (hybrids of the A and C stocks) and DBC mice (obtained by mating DIF_1 hybrids to the D stock). The polymer, polyvinylpyrrolidone (PVP) was used, in the form of Periston N (PVPN), having a molecular weight about 12,600. The mortality rates induced by endotoxin were modified favorably by PVPN in ABC mice, but not in DBC mice. It may be concluded that PVPN beneficially influences survival from Brucella endotoxin in at least one population of hybrid mice. The observation that another population of mice did not gain appreciable benefit from the same dose of PVPN may deserve further studies aimed at elucidating the mechanisms of host response to this agent.

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Fibrinolytic and Coagulant Activities of Certain Snake Venoms and Proteases.* (22647)

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In a search for an ideal agent for the intravascular dissolution of thrombi and emboli in man, investigators have studied heparin(1,2, 3), coumarins(4), trypsin(5), chymotrypsin (3), streptokinase(6), staphylokinase(7), and plasmin(8). Certain snake venoms have long been known to be fibrinolytic *in vitro*. In addition, many of these are also known to contain other proteins with coagulant, hemolytic, neurotoxic or other properties which would contraindicate their clinical use. A survey of available snake venoms was undertaken to compare their *in vitro* lytic actions against fibrinogen, fibrin, formed fibrin and formed whole blood clots with various other proteolytic agents. Coagulant and hemolytic properties were also studied.

Materials. Bovine fibrinogen was a 1% solution of Armour's Fraction I. Human fibrinogen was a crude fraction prepared by 25% saturation of human plasma with ammonium sulfate, collection of the precipitate, dialysis, and adjustment of the clottable protein concentration to 0.5%. Plasmin was a spontaneously activated dog serum unglobulin fraction(9). Plasminogen was prepared from human barium sulfate adsorbed plasma by 20-fold dilution, adjustment of pH to 5.3, collection of precipitate and dissolution in a volume of 0.85% NaCl equal to 1/10 the original plasma volume. The fibrinogen was removed as fibrin after addition of a trace of thrombin (1 unit/10 ml). Streptokinase (Varidase, supplied through the courtesy of Lederle Laboratories) was dissolved in 0.85% NaCl to contain 200 units per ml. Uroprotease was prepared from human urine as described by Celander and Guest(10). Trypsin and chymotrypsin (crystalline, courtesy Armour Laboratories) were 0.1% solutions in 0.85% NaCl. Ficin (Delta), bromelain (courtesy Pineapple Research Institute of Hawaii), papain (Worthington), and pepsin (Worthington) were prepared as 1% solutions in 0.85% NaCl. Snake venoms (supplied through the courtesy of Wyeth and Co.; Ross Allen's Reptile Institute; Burroughs, Wellcome; and Hynson, Wescott and Dunning, Inc.) were dissolved as 1% solutions in 0.85% NaCl. *Stypven* (Burroughs, Wellcome) was used as a 0.2% solution. Thrombin (Thrombin, Topical, Parke Davis) was prepared to contain 100 units per ml.

Methods. Fibrinolysis was estimated by observing lysis time in a clot containing 0.2 ml material to be tested, 0.2 ml fibrinogen, 0.2 ml borate buffer (pH 7.6) and 0.05 ml thrombin. Intimate mixing of the lytic agent and fibrin was obtained by a 2-tube mixing method(11), in which the fibrinogen and buffer were poured into the lytic agent and thrombin, at zero time, and subsequently poured back and forth three times. *Formed clot lysis. Fibrin*: Human fibrinogen clots

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were prepared by adding 0.05 ml thrombin to 0.2 ml fibrinogen, mixing and allowing the clot to stand 15 min. The clot was freed from the tube by a sharp rap and suspended in 0.2 ml material plus 0.2 ml buffer. Lysis time was measured from time of addition of material to time of complete disappearance of the clot. *Whole blood*: 0.2 ml of fresh human blood was pipetted into each of a series of tubes, clotting and retraction allowed to proceed for 1 hour at 37°C and 0.2 ml of the test material added. *Fibrinogenolysis* was tested by incubating 1.0 ml material with 1.0 ml human fibrinogen and testing at intervals for residual fibrinogen by adding 0.2 ml aliquots to 0.05 ml thrombin. The time at which no clot formed was taken as the fibrinogenolysis time.

Thrombic activity was measured by determining the clotting time after addition of 0.1 ml material to 0.1 ml human fibrinogen. *Thromboplastic* activity was determined, in materials which were not thrombic, by substituting 0.1 ml material for thromboplastin in a prothrombin time test. *Hemolytic* and *hemagglutinating* activities were observed in tubes containing 0.2 ml material and 0.2 ml 1% dilution of human whole blood. All procedures were carried out at 37°C.

Results. Actual lysis times observed in the various fibrinolytic systems are shown in Table I. Venoms of the Crotalus, Bothrops and Agkistrodon groups were fibrinolytic, while those of the Cobras (crude Cobra, *N. naja, N. flava*) were not. *V. russellii* was fibrinolytic but inactive against the formed clots. *Stypven,* made from *V. russellii,* was inactive. All of the other agents were fibrinolytic except pepsin, plasminogen alone, and streptokinase in the bovine system. Ficin, bromelain and papain were the only ones with significant activity in the whole blood clots. When trypsin and chymotrypsin were tested as 1% rather than 0.1% solutions, no clots were formed in the fibrinolytic systems and the whole blood clots were lysed in 1 to 2 hours.

Inability of some of the preparations to lyse whole blood clots might depend upon the sensitivity of the enzymes to the serum antiprotease present in the whole blood system.

TABLE I. Fibrinolytic Activities.

	Fibrinolysis (min.)		Clot lysis (min.)	
	Bovine fibrin	Human fibrin	Human fibrin	Human blood
C. basiliscus	<1	5	48	180
C. atrox	3	5	90	62
C. horridus	5	19	151	480
C. adamanteus	15	21	280	480
C. hellerii	NC	8	480	480
C. terrificus	69	>120	1080	>1080
B. neuweidii	4	3	48	68
B. neuweidii 0.1%				>1080
B. jararaca	5	4	65	66
B. atrox	7	7	90	180
B. alternatus	20	21	155	480
A. piscivorus	4	8	65	65
A. mokasen	6	7	65	180
Crude Cobra	>120	>120	>1080	>1080
N. naja	>120	>120	>1080	>1080
N. flava	>120	>120	>1080	>1080
V. russellii	<1	5	>1080	>1080
'Stypven' 0.2%	>120	>120	>1080	>1080
Plasmin (dog)	4	5	148	>1080
Plasminogen (human)	>120	>120	>1080	>1080
Streptokinase (SK)	>120	5	142	>1080
Uroprotease (UP)	8	10	92	>1080
Plasminogen + SK	2	2	57	>1080
Plasminogen + UP	12	9	84	>1080
Trypsin 1 %	<1	<1	25	72
" .1%	<1	<1	28	>1080
Chymotrypsin 1 %	1	1	25	120
" .1%	1	2	32	1080
Ficin	5	31	1080	240
Bromelain	<1	2	280	540
Papain	2	2	900	160
Pepsin	>120	>120	>1080	>1080
Saline	>120	>120	>1080	>1080

Therefore, additional tests were performed by incubating the various agents with human serum and measuring residual lytic activity. The active snake venoms, ficin, bromelain and papain were inhibited far less than plasmin, uroprotease, and streptokinase-plasminogen. Trypsin and chymotrypsin showed variable activity: either increased or decreased, depending upon time of incubation and strengths of serum and enzyme, suggesting that two processes were occurring simultaneously: inhibition and activation of the serum plasminogen.

Thrombic activity, *i.e.* ability to gel fibrinogen, was found in 8 venoms and papain (Table II). Only trypsin, *V. russellii,* and

TABLE II. Toxic Activities.

Agent	Thrombic (sec.)	Thrombo-plastic (sec.)	Fibrino-genolytic (min.)	Hemolytic	Hemaggluti-nating
C. basiliscus	>600	>600	5	0	0
C. atrox	>600	>600	5	0	+
C. horridus	21.0	—	—	0	0
C. adamanteus	10.0	—	—	0	0
C. hellerii	45.0	—	—	0	0
C. terrificus	16.6	—	—	0	0
B. neuweidii	10.8	—	—	0	+
B. jararaca	18.4	—	—	0	0
B. atrox	11	—	—	0	0
B. alternatus	21.4	—	—	0	0
A. piscivorus	>600	>600	15	0	+
A. mokasen	>600	>600	10	0	+
Crude cobra	>600	>600	10	+	0
N. naja	>600	>600	10	+	0
N. flava	>600	>600	5	+	0
V. russellii	>600	30	25	0	0
"Stypven"	>600	19.0	>60	0	0
Plasmin (dog)	>600	100	>60	0	0
Plasminogen (human)	>600	56.5	>60	0	0
Streptokinase (SK)	>600	100	26	0	0
Uroprotease (UP)	>600	100	>60	0	0
Plasminogen + SK	>600	100	25	0	0
" + UP	>600	100	60	0	0
Trypsin .1%	>600	12.8	<¼	0	0
Chymotrypsin .1%	>600	68	<¼	0	0
Ficin	>600	63	¾	+	0
Bromelain	>600	>600	15	+	0
Papain	25.0	—	—	0	0
Pepsin	>600	100	>60	+	0
.85% NaCl	>600	95	>60	0	0

its derivative *Stypven* were thromboplastic. Certain snake venoms appeared anti-coagulant, *i.e.* thromboplastin time longer than that of saline. In each case this appeared due to fibrinogenolysis.

All of the snake venoms that could be tested (those which were not thrombic) were fibrinogenolytic, as were the other fibrinolytic enzymes. The cobra venoms were remarkable in being fibrinogenolytic but not fibrinolytic. Hemolysis was produced by the cobra venoms, ficin, bromelain and pepsin. Agglutination of human red cells, seen grossly and confirmed microscopically, was caused by *C. atrox, B. neuweidii, A. piscivorus* and *A. mokasen.*

The possibility that strongly fibrinogenolytic venoms showing no thrombic activity might actually be thrombic as well at a lower concentration was investigated. Fibrinogen was incubated with various strengths of three such venoms (*A. mokasen, A. piscivorus, C. basiliscus*) from 1% down to .00002%, the latter figure being far below the concentration necessary for fibrinogenolysis to be evident. At none of these strengths was thrombic activity apparent.

Discussion. Fontana, in 1787(12), noted the blood to remain fluid in animals dead of viper bite. Since then various venoms have been found to exhibit various activities on the coagulation and fibrinolytic mechanisms(13, 14,15). Therapeutic use in hemorrhagic diseases has not been encouraging. Other properties of venoms, including hemolysins(16), neurotoxins(17) and hyaluronidase(18) have been the subject of many investigations. The data here presented indicate that a number of snake venoms actively lyse human blood clots and appear to have a certain theoretical advantage over the more common fibrinolytic agents in that they are less inhibited by human serum antiprotease. However, these venoms are all either fibrinogenolytic, thrombic,

thromboplastic, hemolytic or hemagglutinating, thus contraindicating their clinical use in crude form. Fractionation of whole venoms has been reported(19,20) and it seems conceivable that a pure fibrinolytic fraction, devoid of toxic activities, might be separated and prove of therapeutic value.

Summary. Of the 16 snake venoms studied, 11 actively lysed human blood clots. However, only one of these, *C. basiliscus,* was devoid of thrombic, hemolytic, and hemagglutinating properties. This venom was fibrinogenolytic as well as fibrinolytic. The possible therapeutic use of certain venoms as dissolving agents for intravascular clots presents a theoretical advantage over most other fibrinolytic agents in that their fibrinolytic activity is not readily inhibited by human serum. Fractionation of a pure fibrinolytic principle may be possible.

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Effect of Thyroxine on Liver Tyrosine-glutamic Acid Transaminase. (22648)

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In the course of studying the effects of different hormonal states and hormone administration upon activity of several rat liver enzymes it was observed that dietary treatment with 1% thyroid for 8 days or more resulted in marked inhibition of liver tyrosine oxidation(1). Furthermore, the degree of inhibition was equally significant in normal animals and in animals under conditions of varying hormonal state, whereas certain of the other enzymes were affected by thyroid only under restricted conditions. This suggested a direct effect of the hormone* upon the tyrosine oxidase system. Since low dietary protein decreased activity of homogenate preparations (1) and inanition has been reported to enhance tyrosine oxidation in soluble preparations(2) this effect was further investigated

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* Reference to thyroxine hormone means thyroxine itself or whatever active form to which it may be converted.

using *in vitro* technics.

Methods and materials. Mature Long-Evans strain male rats weighing between 200 and 400 g were used as a source of liver tissue. The enzyme was prepared by homogenizing fresh, chilled liver with 2 volumes of cold 0.14 M KCl and centrifuging at 30,000 rpm for 45 minutes in a refrigerated Spinco centrifuge with a No. 40 rotor. The clear supernatant was used as the enzyme preparation. Enzyme activity was measured by use of an automatic recording Beckman spectrophotometer with a thermostat-controlled heating chamber at 37°C. Tracings from the recorder measured changes in optical density at 320 mμ (U. V. Light) which measured appearance of the enol form of p-hydroxyphenyl pyruvate (HPP) and tautomerization of the keto form to the enol form of HPP by tautomerase(3). Keto HPP shows end absorption in U.V. light while the enol form absorbs maximally at 300 mμ. The transaminase-tautomerase system included: 6 μM tyrosine in phosphate buffer(3), 0.1 ml of 0.1 M α-keto glutarate at pH 7.0, 1 ml of 0.2 M phosphate buffer at pH 7.0, 0.3 ml of soluble enzyme preparation (pH 7.0) and water to make a total volume of 3.6 ml. The final pH of the system was 7.0. The system used to measure tautomerase activity included: 6 μM keto HPP, pH 6.8, 1.0 ml 0.2 M phosphate buffer at pH 7.0, 0.3 ml enzyme and water to make 3.6 ml. L-thyroxine was dissolved in 0.02 N NaOH (pH 8)(4) and was added to the system in 0.1 ml and pre-incubated with the enzyme for 2 minutes at 25°C before addition of substrate, however, this procedure was not necessary to obtain the hormone action. The reaction was carried out in 1 cm quartz cells and the complete system was set to zero optical density immediately after the addition of the substrate. Either tautomerase or transaminase-tautomerase activity has been expressed as the change in optical density at 320 mμ in 10 min. (Δ O.D. 320 mμ/10 min.). One ml of the enzyme contained approximately 1.8 mg Kjeldahl nitrogen.

Results. When the fresh enzyme preparation was used to measure enol HPP accumulation with tyrosine as substrate, equilibrium enol HPP formation was reached in about 10 min. Further oxidation of HPP by oxidative enzymes could be seen in 20 minutes when enol HPP had nearly disappeared. In the presence of 10^{-4} M thyroxine the overall reaction rate was reduced by about 60%. Since Knox and Knox(6) have demonstrated that the transaminase reaction limited the overall rate of the system converting tyrosine to fumarate and acetoacetate and from kinetic studies of this system measuring QO_2(1) it appeared that the site of inhibition by thyroxine must be early in the tyrosine metabolizing sequence. After 48 hours of aging the enzyme at 5°C, further oxidation of enol HPP could no longer be detected during 60 minutes of reaction with tyrosine and α-ketoglutarate in the system as substrates. This meant that the residual activities were those of transaminase (E_1) and tautomerase (E_2) whose activity had not decreased significantly during this time:

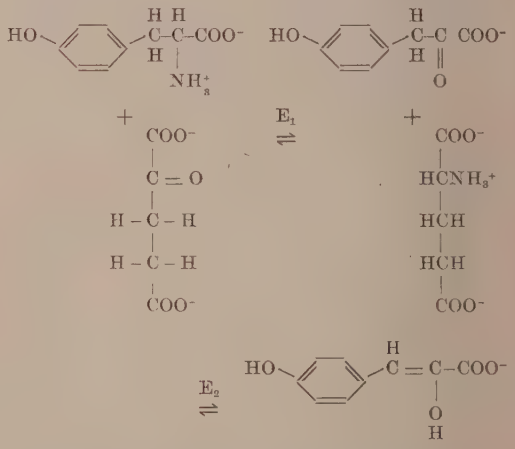

Table I shows the effect of increasing concentrations of thyroxine on coupled transaminase-

TABLE I. Effect of Increasing Concentrations of Thyroxine upon Tyrosine Glutamic Acid Transaminase-Tautomerase Activity.

Enzyme* concentration (mg N)	L-Thyroxine concentration (M)	Transaminase-tautomerase activity (△ O.D. 320 mμ/10 min.)
.54	—	.274
"	5.10^{-5}	.121
"	1.10^{-4}	.075
"	2.10^{-4}	.051

* 3 day aged enzyme.

TABLE II. Effect of Thyroxine upon Activities of Coupled Transaminase-Tautomerase and Tautomerase.

Thyroxine conc. (M)	Substrate (6 μM)	Enzyme activity* ($\triangle$ O.D. 320 mμ/10 min.)	% inhibition
None	L-tyrosine	.220	—
10^{-4}	"	.082	63
None	keto HPP	.330	—
10^{-4}	"	.300	9

* 3 day aged enzyme.

tautomerase activity. Since the results showed significant effects upon this system it was essential to determine which enzyme was being inhibited by the hormone.

Table II shows the effect of added thyroxine to the coupled enzyme system and also upon the tautomerase reaction which could be isolated from E_1 by omitting α-ketoglutarate from the system and supplying keto HPP as substrate. The formation of enol HPP was then measured in the aged preparation which was free of detectable oxidative steps during the experimental period. While only a small inhibition of E_2 activity was noted in the presence of thyroxine a large inhibition occurred in the E_1E_2 reaction which indicated that the site of thyroxine action occurred at the transaminase level. In addition, this inhibition appeared to be non-competitive in nature as determined by the method of Ackermann and Potter(5).

Discussion. The possibility that thyroxine may act as an inhibitor of other transaminating enzymes is being investigated. The effective levels of thyroxine indicate that this reaction may be of physiological importance. *In vivo* data(1) on tyrosine oxidation add credence to this point. The significance of such a mechanism in regard to protein synthesis is obvious. Previous work with diiodotyrosine(1) indicates that this substance inhibits at the substrate level (competitive inhibition) while thyroxine is more effective at lower concentrations and appears to be binding the enzyme (non-competitive inhibition) perhaps analogous to thyroglobulin formation. Binding of thyroxine has been observed to occur in serum by a "thyroxine binding protein"(7) presumably of the G_1 globulin fraction. A report of *in vivo* studies and mechanism of inhibition will appear later.

Summary. Thyroxine in concentrations as low as 5.10^{-5}M has been shown to markedly inhibit transaminase-tautomerase activity of the liver tyrosine oxidase system. The transaminase enzyme appears to be the most directly involved in the inhibition as determined by difference studies.

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Growth and Regeneration of *Schistosoma mansoni in vitro*.*† (22649)

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The development of technics for the cultivation of the parasitic trematodes of man has progressed slowly. Studies on the most important human pathogens in this group, the schistosomes, have dealt primarily with the longevity of adult worms under artificial conditions in media containing serum and with attempts to define the constituents of serum essential for their survival. *Schistosoma japonicum* has survived *in vitro* for as long as 82 days in a rabbit serum medium(1), and for almost 5 months in horse serum-Ringer's solution containing washed red cells(2). Mature *S. mansoni* have been maintained for 18 days in ox serum(3), and for 2 months in horse serum(4). Egg production has been reported in a human serum system(5). With a chemically defined medium, a survival time for *S. mansoni* of 5 days has been noted(6).

Smyth(7) has emphasized that in studies on helminths *in vitro* criteria for demonstration of "growth" as contrasted to mere "survival" need to be developed. In this paper we will present evidence that growth of immature schistosomes maintained *in vitro* occurred, as manifested by an increase in size and by regeneration of amputated portions of certain of the worms.

Materials and methods. Specimens of immature *S. mansoni* were obtained employing aseptic technics from the portal system of white mice infected percutaneously in our laboratory. In addition, schistosomulae were recovered from peritoneal washings and from washings of liver fragments of mice inoculated with cercariae by the intraperitoneal route; these infected mice were supplied through the courtesy of Dr. Henry van der Schalie of the University of Michigan.

Infected mice were lightly etherized and then killed by cervical fracture. The peritoneal cavity was flushed with culture fluid (CF) in the case of mice infected by inoculation. The portal vessels and liver were then removed to separate Petri dishes containing CF and worms recovered by the teasing apart of the tissues under a dissecting microscope. Mice that had been infected by the percutaneous route were given 0.4 ml of heparin sodium (Liquaemin, Organon) intraperitoneally 5 minutes before being sacrificed.

The worms were transferred with Pasteur pipettes through several changes of fluid and then placed singly or in pairs in rubber-stoppered or screw-capped test tubes (15 x 150 mm) containing 1.4 ml of CF. The culture fluid was that used by us for maintenance of human cells *in vitro,* with the single omission of soybean trypsin inhibitor. It consisted of 45% bovine amniotic fluid(8), 45% Hanks' balanced salt solution(9), 5% inactivated horse serum, and 5% beef embryo extract, with penicillin, streptomycin and phenol red as described(8). Whenever necessary, the pH was adjusted to about 7.4 prior to use by gassing with 5% CO_2 in air. Cultures were maintained in an upright position at 37°C. At 2- to 4-day intervals, the CF was withdrawn and fresh medium substituted. The basic medium was supplemented in certain experiments by the addition to each tube at weekly intervals of fresh washed mouse red blood cells. After each addition, the erythrocytes were left in the cultures for 2 to 4 days and then removed. Cells for this purpose were obtained from young female white mice; such mice were heparinized, sacrificed as described above, and bled from the exposed heart. The cells were washed and centrifuged 3 times in CF. Approximately 0.3 ml of packed cells was resuspended in 2.0 ml of medium and 2 drops of the resulting suspension added to each culture. Final concentration

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† We are indebted to Mr. John Carabitses for the preparation of the photomicrographs.

of red cells in the culture medium was determined to be equivalent to a hematocrit of 3 to 5.

All subsequent manipulations of the cultures, with exception of photographic procedures, were carried out in a warm room at 32°C. The schistosomes were observed daily under a compound or dissecting microscope. This procedure was facilitated by gently rotating the culture in an almost horizontal position until the worm adhered to the tube wall out of the fluid. Serial camera lucida drawings were made in order to document changes in size. Inasmuch as constant contraction and extension of the worms was occurring, exact determination of length was difficult. Therefore, at each observation period 3 to 6 outline drawings were made per worm. A "map measurer" calibrated by mensuration of the projected image of a slide micrometer, was run along the axis of each outline. Length was arbitrarily derived by averaging sets of measurements. Serial drawings of worms less than 0.5 mm could not be obtained, since worms of this size could not regularly be induced to adhere to the wall of the culture tube. In a number of instances, interval photographs were taken. Tracings of these photographs were transferred to graph paper for calculation of total area as a measurement of body growth. Results generally corroborated data on length as obtained by multiple camera lucida drawings.

The CF, removed from each tube at the first change of medium, was cultured in thioglycollate broth and on Sabouraud's agar to reveal bacterial or fungal contaminants. Similar control cultures were repeated at intervals. No bacterial contamination was encountered, but growth of a mold resulted in loss of 3 worms.

FIG. 1. Male schistosome (#905) recovered from mouse on 16th day of infection and photographed on 11th day *in vitro*. Length approx. 2 mm.

Results. A total of 78 schistosomes was studied *in vitro* for various periods of time. Although all worms showed some increase in length, irrespective of initial size, the survival period *in vitro* of worms measuring less than 0.5 mm was limited. The results of experiments with schistosomulae recovered from the peritoneal cavity of mice 6 to 10 days after injection of cercariae are summarized in Table I. In this series, erythrocytes were not added to the medium. A similar experiment was initiated using 11 worms obtained from mice on the 16th day of infection. For 2 weeks these were maintained in individual cultures on CF alone; at that point 5 of the worms were given red cells which appeared to increase activity and prolong slightly their survival. Two worms were alive after 35 days in culture, at which time the experiment was terminated; one, a female (#911) had increased in length from 0.98 mm to 4.58 mm, while the other, a male (#905) had increased from 1.52 to 4.08 mm. These schistosomes are pictured in Fig. 1 and 2. The results of serial measurements on the 16-day-old schistosomulae are summarized in Table II.

Immature schistosomes, derived from mice 30 or more days after percutaneous infection,

TABLE I. Survival of Schistosomulae *In Vitro*.

No. of worms in group	Time in days between infection of mice and recovery of worms	Original length (mm)*	Final length (mm)*	Avg days survival *in vitro*
6	6	.12–.25	.25– .50	3.3
6	9	.15	.50– .75	10.5
4	10	.25	.75–1.2	10.7

* Length estimated; serial measurements not done.

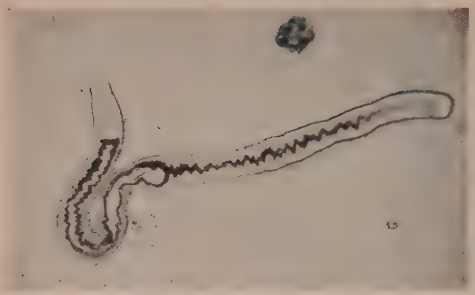

FIG. 2. Female schistosome (#911) recovered from mouse on 16th day of infection; photographed on 24th day *in vitro* and 24 hr after red cells had been added to the medium. Length 2.4 mm.

and having an initial length exceeding 1.5 mm, generally survived for many weeks on CF supplemented with red cells. Thus, 40 trematodes in this category remained alive and displayed vigorous feeding activity for periods as long as 87 days. Serial measurements on 3 male worms and 1 female in this group are presented graphically in Fig. 3. Accelerated somatic movement and peristaltic activity usually followed ingestion of the added red cells. After an exposure of 8 to 24 hours to the erythrocytes, ceca previously almost devoid of pigment would become distended with black material; usually such pigment disappeared within 48 hours following the withdrawal of red cells from the system.

Three pairs of larger worms (3-5 mm), each pair consisting of a male and a female, were placed in separate tubes. All began to copulate *in vitro*. In one case this attitude was maintained for 2 months, while the other pairs parted and rejoined at irregular intervals. No eggs were produced.

No systematic study was undertaken of the ability of schistosomes to regenerate amputated portions, but observations were made on a limited number of worms accidentally traumatized during recovery procedures. Four worms that lacked the posterior ⅓ to ½ of the body were noted to regenerate the missing part *in vitro* within 10 to 20 days. Ceca, cut just proximal to the point of union, were seen to unite and form the usual single posterior trunk during the regenerative period. In one specimen, a large cyst-like bladder developed in the regenerating posterior third. Although

TABLE II. Survival and Growth *In Vitro* of 16-Day Schistosomulae.

Worm No.	Original length (mm)*	Final length (mm)*	% size increase	No. days survival
901	1.0	1.23	23	21
902	.94	1.01	7	26
904	.46	.61	33	25
905	1.52	4.08	168	(35)†
906	.62	.92	48	23
908	.67	1.09	63	25
909	.63	.87	38	17
910	.54	.67	24	17
911	.98	4.58	367	(35)†
912	.41	.61	49	17
913	.58	.61	5	17

* Averages derived from camera lucida drawings.

† ( ) = alive at end of experiment.

the excretory system remained imperfect, this individual survived during the 75-day observation period. Two schistosomes lacking the anterior third of the body were observed for 23 days, during which time there was no noticeable diminution of activity.

Discussion. The results of these preliminary studies indicate that a nutrient medium suitable for the cultivation of mammalian cells will also induce growth of *S. mansoni in vitro* as evidenced by an increase in length and by the regeneration of somatic amputations. That this nutrient system is not opti-

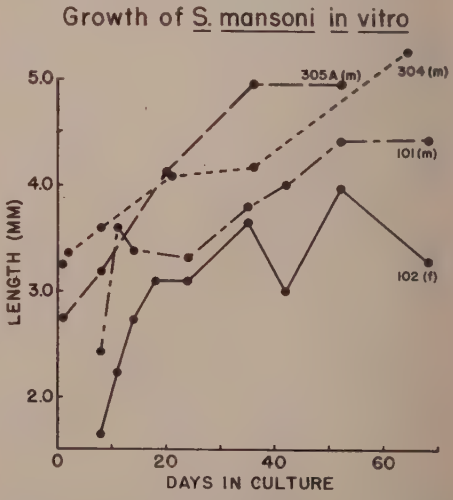

FIG. 3. Serial measurements derived from camera lucida drawings of 4 schistosomes maintained *in vitro* on CF supplemented with red cells.

mal is clearly indicated by the limited survival periods of the small specimens. The apparent stimulatory effect of ingested red cells on the larger worms requires further investigation. It is to be noted that Moore and Meleney(10) have suggested that the defective maturation of *S. mansoni* in the peritoneal cavity of the mouse may be a reflection of the absence of blood upon which to feed. Although not yet ideal, the *in vitro* system here described offers a convenient means for the direct and prolonged observation of feeding schistosomes. The technic should, therefore, prove useful for a variety of experimental purposes, as well as offering a baseline for the development of improved media in the future.

Summary. Preliminary investigations on the growth of *Schistosoma mansoni in vitro* are reported. In a nutrient medium composed of bovine amniotic fluid, beef embryo extract, and horse serum, schistosomulae generally showed evidence of growth. The duration of survival was directly related to initial size. Worms that initially approximated 1.0 mm or greater in length were found to survive many weeks *in vitro;* the survival period of smaller schistosomulae was of much shorter duration. The addition of red cell suspensions to the basic medium was followed by active feeding and accelerated somatic movements. Growth of the schistosomes *in vitro* was manifested by increases in length of male worms to a maximum of 168%, and of female worms to 367%, and also by the observed regeneration of amputated portions of certain of the worms.

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Action of Thyroid Hormones on Brain Metabolism of Newborn Rats. (22650)

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Investigations have shown that the metabolism of the brain is better protected against interference, especially of the thyroid hormone, than other tissues(1a). It was reported(1) that thyrotrophic hormone can increase the brain oxidation of hypophysectomised rats, although it can seldom influence that of normal adult rats. It seemed that after hypophysectomy a regulating mechanism, which is responsible for the constancy of the brain metabolism, is disturbed. During the last few years it seemed promising to continue these experiments on animals whose anterior pituitary function was not yet fully developed. The brain of newborn rats seemed also to be particularly interesting owing to the extraordinary resistance of these animals to anoxaemia which enables them to survive in a carbon monoxide atmosphere(2).

Contrary to previous experiences with adult animals these investigations showed some significant changes after treatment with thyroid hormones.

Methods. Litters of Wistar rats were 5 to 11 days old at investigation, and weighed 8 to 20 g each. Each litter was divided equally,

TABLE I. O_2 Consumption of Cerebral Cortex of Newborn Rats after Thyroxine Treatment.

		Controls (saline treated)		Thyroxine treated animals				
				Thyroxine treatment				
Litter No.	Age in days	No. of animals	Avg O_2 consumption/ hr/100 mg fresh tissue, $mm^3 \pm$ S.D.	No. of animals	Total dose, mg	No. of single daily doses	Avg O_2 consumption/ hr/100 mg fresh tissue, $mm^3 \pm$ S.D.	% differences as compared with controls
I	8	4	81.5 ± 5.5	4	.15	1	84.7 ± 7.5	+ 3.9
II	7	6	76.5 ± 7.0	6	.15	1	86.2 ± 5.8	+12.7*
III	8	4	84.0 ± 3.7	5	.15	2	93.6 ± 9.7	+11.4
IV	9	4	89.5 ± 5.7	4	.15	2	99.2 ± 1.7	+10.8*
V	8	4	78.0 ± 4.1	5	.15	3	91.2 ± 6.5	+16.9†
VI	8	6	97.2 ± 2.6	6	.25	3	109.8 ± 4.7	+13.0†
VII	9	5	92.2 ± 2.4	7	.25	5	102.1 ± 6.8	+10.7†
VIII	9	3	88.3 ± 2.6	3	.25	5	105.0 ± 1.7	+18.9†

* Significant at P 0.05. † Significant at P 0.01.

TABLE II. O_2 Consumption of Cerebral Cortex of Newborn Rats after Treatment with Thyroxine and Triiodothyronine.

		Treatment control animals (saline treated)		.1 mg triiodothyronine, daily for 4 days			.1 mg thyroxine, daily for 4 days		
Litter No.	Age in days	No. of animals	mm^3 O_2/hr/ 100 mg fresh tissue, mean $\pm$ S.D.	No. of animals	mm^3 O_2/hr/ 100 mg fresh tissue, mean $\pm$ S.D.	% differences as compared with controls	No. of animals	mm^3 O_2/hr/ 100 mg fresh tissue, mean $\pm$ S.D.	% differences as compared with controls
1	10	5	85.2 ± 6.3	4	96.8 ± 15.2	+13.5			
2	10	4	83.2 ± 4.9	4	110.0 ± 4.8	+32.1*			
3, 4, 5 comb.	10	7	104.7 ± 6.5	6	114.8 ± 3.1	+11.0*	7	115.4 ± 8.7	+11.0*
							Idem for 6 days		
6	10	2	99.5 ± 6.4	2	126.5 ± 9.2	+27.1	3	124.0 ± 5.6	+24.6†

* Significant at P 0.01. † Significant at P 0.05.

TABLE III. O_2 Consumption of Cerebral Cortex of Newborn Rats after Treatment with Thyrotrophic Hormone.

		Controls (saline treated)		Animals treated with thyrotrophic hormone				
				T.S.H. treatment				
Litter No.	Age in days	No. of animals	Avg O_2 consumption/ hr/100 mg fresh tissue, $mm^3 \pm$ S.D.	No. of animals	Total dose, i.u.	No. of single daily doses	Avg O_2 consumption/ hr/100 mg fresh tissue, $mm^3 \pm$ S.D.	% differences as compared with controls
I	8	3	92.7 ± 6.0	4	1.6	3	98.5 ± 10.1	+ 6.3
II	8	4	91.2 ± 4.8	5	1.6	3	96.8 ± 6.5	+ 6.1
III	8	4	90.7 ± 5.2	5	1.6	7	104.6 ± 4.6	+15.3*

* Significant at P 0.01.

TABLE IV. O_2 Consumption of Cerebellum of Newborn Rats after Thyroxine Treatment.

		Controls (saline treated)		Thyroxine treated animals				
				Thyroxine treatment				
Litter No.	Age in days	No. of animals	Avg O_2 consumption/ hr/100 mg fresh tissue, $mm^3 \pm$ S.D.	No. of animals	Total dose, mg	No. of single daily doses	Avg O_2 consumption/ hr/100 mg fresh tissue, $mm^3 \pm$ S.D.	% differences as compared with controls
I	8	5	133.4 ± 15.7	6	.25	5	145.8 ± 12.6	+ 9.3
II	8	4	127.0 ± 11.1	4	.25	5	145.0 ± 21.8	+14.2
III	11	4	121.7 ± 23.6	4	.15	3	151.5 ± 11.8	+24.5

TABLE V. % Dry Substance of Cerebral Cortex, Cerebellum and Medulla Oblongata of Newborn Rats Treated with Thyroxine, Triiodothyronine and Thyrotrophic Hormone.

Litter No.	Age in days	No. of animals	% dry substance, mean ± S.D.			No. of animals	% dry substance, mean ± S.D.			% difference as compared with controls		
			Cortex	Cerebellum	Medulla oblongata		Cortex	Cerebellum	Medulla oblongata	Cortex	Cerebellum	Medulla oblongata
			Control animals, saline treated				.05 mg thyroxin daily, 3 days					
I	5	4	12.3 ± .25	12.7 ± .54	12.2 ± .27	4	12.8 ± .24	14.1 ± .27	12.9 ± .41	+5.9*	+11.5†	+ 5.6*
II	8	5	12.4 ± .25	13.2 ± .19	14.1 ± .32	4	13.2 ± .22	14.7 ± .20	14.9 ± .36	+6.4†	+11.0†	+ 5.8†
III	11	4	14.15± .31	14.3 ± .46	15.2 ± .36	4	14.7 ± .30	15.4 ± .38	16.8 ± .54	+3.5	+ 7.7†	+10.3†
							.1 mg tri-iodothyronine daily, 4 days					
IV	6	3	12.2 ± .12	13.9 ± .61	13.9 ± .73	4	13.2 ± .36	14.6 ± .22	14.3 ± .29	+8.7†	+ 4.9	+ 2.9
V	11	3	14.1 ± .33	15.1 ± .30	15.1 ± .29	3	15.0 ± .52	16.1 ± .21	16.3 ± .35	+6.1	+ 6.5†	+ 8.0†
							.2 i.u. thyrotrophic hormone daily, 7 days					
VI	10	5	13.6 ± .43	14.9 ± .20	15.1 ± .35	4	14.3 ± .15	15.7 ± .17	15.5 ± .24	+5.7†	+ 5.2†	+ 3.0
VII	10	6	13.2 ± .15	14.3 ± .29	14.6 ± .39	5	14.3 ± .49	15.1 ± .42	15.6 ± .25	+8.4†	+ 5.6†	+ 6.7†

* Significant at P 0.05. † Significant at P 0.01.

one half receiving daily injections of either thyroxine, thyrotrophic hormone, or triiodothyronine, and the other, or control half, receiving normal saline. The litters remained with the mother throughout the period of treatment. The animals were killed by decapitation, the brain quickly removed and, in order to expedite the start of the experiment, small tissue snippets were cut with a pair of fine eye scissors. These snippets were sufficiently thin to show the same oxygen consumption as brain slices, as had been established in a number of preliminary investigations. The snippets were weighed on a torsion balance with a capacity of 100 mg. Ninety to 95 mg of tissue snippets were investigated in conical Warburg vessels. Krebs Phosphate Ringer containing 0.2% glucose was used as suspension fluid.

Results. In Table I changes in the oxygen consumption of the cerebral cortex after thyroxine treatment are recorded. There seems to be some relation between duration of treatment and extent of increase in oxygen consumption.

Table II shows the influence of triiodothyronine on oxygen consumption, and also a comparison between the action of equal weight doses of thyroxine and triiodothyronine. The experiments show that triiodothyronine increases rate of brain respiration in newborn rats, but does not permit a conclusion that it has, in this case, a greater effect than thyroxine.

The influence of thyrotrophic hormone (TSH) on the brain cortex of newborn rats is shown in Table III. A dose of TSH which in 3 doses over 3 days seemed to produce little effect, appeared to produce a larger and statistically significant difference from the controls when given in 7 doses over 7 days.

The cerebellum of the newborn rat has normally a considerably higher oxygen consumption than that of the cortex. Thyroxine appears to raise the oxygen consumption (Table IV). The results however, are not significant owing to the great scatter in the single results.

Changes in the dry substance content of cortex, cerebellum and medulla region were

investigated in 40 litters. A few representative results are given in Table V. This table shows that thyroxine, triiodothyronine and thyrotrophic hormone alike increase the dry substance content of all brain parts. Triiodothyronine does not appear to have a more marked action than thyroxine.

Discussion. It has been shown in these investigations that the oxygen consumption of the brain of the newborn rat can be increased under the influence of thyroid hormones. Thus it is considerably more sensitive to this treatment than the brain of the grown-up animal. It appears feasible that this action is equivalent to an acceleration of a physiological maturation process taking place in the brain. It is known(3) that the oxygen consumption of the brain rises during the first weeks of life.

It would be interesting to know why thyroid hormone acts as described on the brain of the newborn animal and not on that of the adult. It has been recently shown that the blood brain barrier is not yet established in newborn rats against glutamic acid(4) or Cl^{36}, or Thiocyanate(5). It would be tempting to assume that, similarly, during the first few days of life, thyroid substances can more easily contact the brain cells, owing to the fact that the blood brain barrier is not yet fully developed during this time.

Summary. 1. Thyroxine, triiodothyronine and thyrotrophic hormone significantly raise the brain oxidation of newborn rats; the dry substance content is also raised. These changes were regarded as an acceleration of normal maturation processes. 2. It is assumed that the described action of thyroid hormones on the brain of newborn animals is due to a still incompletely developed blood brain barrier.

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In vitro Effect of Soybean Phosphatides on Serum Lipoproteins of Normal and Hyperlipemic Subjects.* (22651)

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The oral administration of soybean lecithin has previously been reported to lower the serum cholesterol of man(1) and cholesterol-fed rabbits(2). The present study was initiated when preliminary experiments revealed that incubation of human serum, after addition of a fat emulsion prepared for intravenous administration, caused a shift of lipid from beta-globulin to alpha-globulin, as determined by paper electrophoresis. Because lecithin was used as a stabilizer in the fat emulsion, this phenomenon was investigated further using soybean phosphatides. The effects of not only the whole phosphatide complex, but also the alcohol-soluble and the alcohol-insoluble fraction were investigated.

Material and methods. 1. *Exp. A—Whole Phosphatide Complex.* A purified extract of natural soybean phosphatides† containing approximately equal quantities of lecithin, cephalin and lipositol was used as follows: Five mg of soybean phosphatides were dissolved in 1 or 2 ml of sera from 6 normal subjects in

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† This compound, Asolectin, was kindly supplied by Mr. J. Eichberg of the American Lecithin Co., Inc.

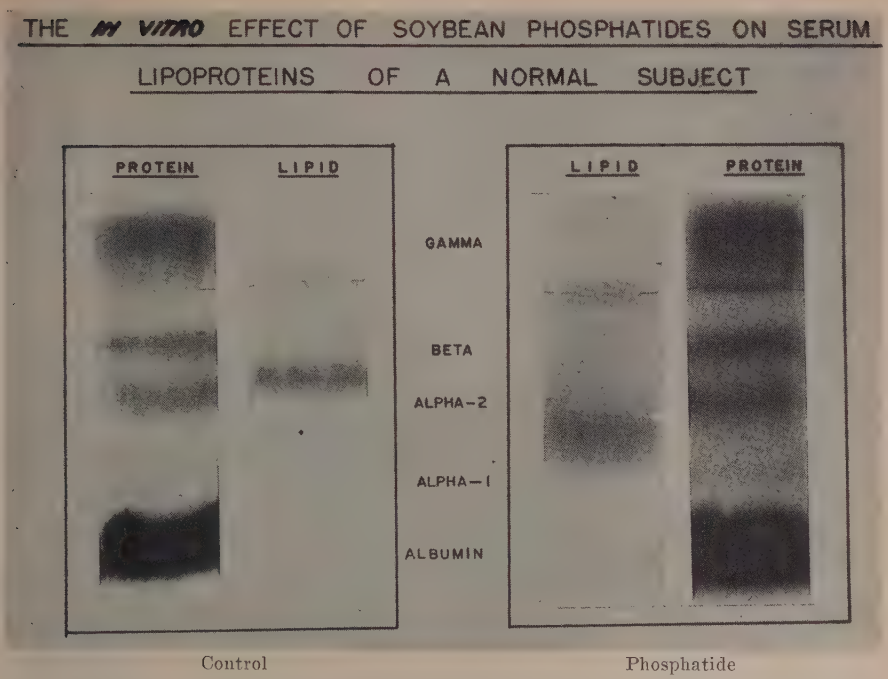

FIG. 1. Effect of incubating the whole phosphatide complex with serum from a normal subject. Lipid stain is red; black and white reproduction does not permit good visualization of the faint alpha lipoprotein band.

the post-absorptive state. In a similar manner, 10 or 20 mg of the phosphatide complex were dissolved in the sera of 5 patients with hyperlipemia.‡ Serum aliquots without added phosphatide served as controls. The control sera and sera with added phosphatide were immediately streaked on filter paper strips for electrophoretic analysis, which was promptly performed with Flynn and deMayo's modification of Durrum's apparatus(3). The serum aliquots of 0.01 or 0.02 ml were streaked for protein or lipid staining respectively. The remaining control and experimental sera were incubated at 38°C for 24 hours, before being subjected to paper electrophoresis. Protein was stained with naphthalene black 12B200 and lipid with oil red O by technics previously described(4). Total lipids were determined gravimetrically (4), and lipid phosphorus by a modified Fiske-SubbaRow procedure(5) in aliquots of serum extracted with Bloor's reagent (alcohol 3 parts/anhydrous ether, 1 part). Cholesterol was determined by Bloor's method(6) on the total lipid residue.

2. *Exp. B-Alcoholic Fractions of the Phosphatide Complex.* The effects of the alcohol-soluble fraction of the soybean phosphatides, consisting of approximately ⅔ lecithin and ⅓ cephalin, and the alcohol-insoluble fraction, consisting of approximately ⅔ lipositol and ⅓ cephalin, on the serum proteins and lipoproteins of 10 normal and 9 hyperlipemic subjects were studied. In 1 ml of each normal serum, 5 mg of each fraction were separately dissolved. Similarly, in 1 ml of each hyperlipemic serum, 10 mg were dissolved. Samples of the resulting 4 mixtures and untreated control sera then were subjected to electrophoretic analysis both before and after incu-

‡ Early in these studies it was found that incubation with 5 mg of the phosphatide had no effect on migration in markedly hyperlipemic sera. When the phosphatide added was increased to 10 or 20 mg, changes in lipoprotein similar to that demonstrated in the normals were noted.

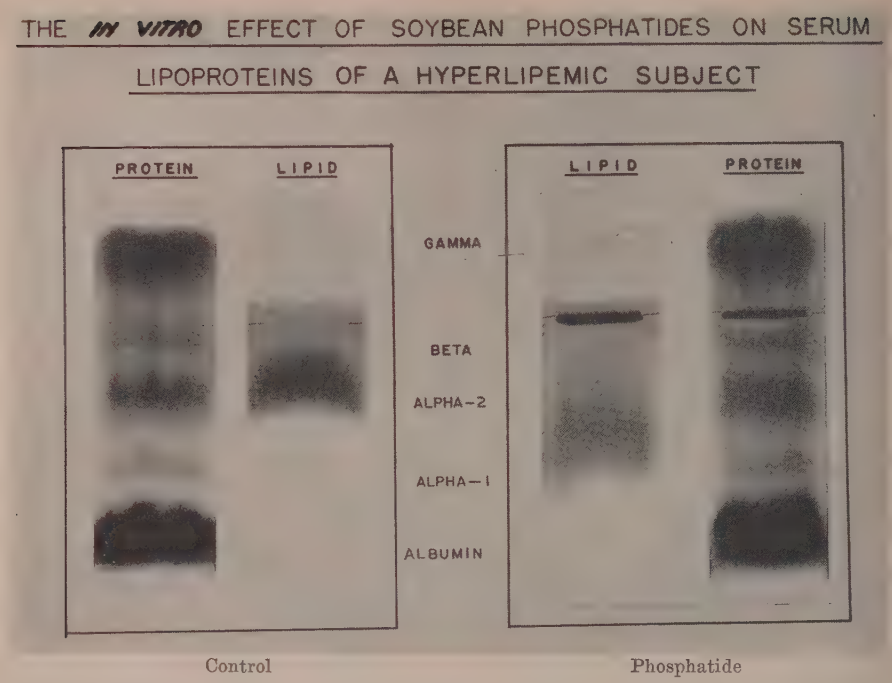

FIG. 2. Effect of incubating the whole phosphatide complex with serum from a hyperlipemic subject.

bation at 38°C for 24 hours. As in Exp. A, all sera were analyzed for total lipid, lipid phosphorus and total cholesterol prior to the experiment to determine degree of lipemia.

3. *Exp. C—Free Fatty Acids.* Free fatty acids were determined by the method of Grossman *et al.*(7) in aliquots of 7 sera incubated without phosphatide or with the alcohol-soluble or the alcohol-insoluble fractions. As in Exp. A, to 1 ml of each normal serum, 5 mg of either phosphatide were added whereas 10 mg were added to 1 ml of each of 2 hyperlipemic sera.

Results. 1. *Exp. A.* With the technic utilized, the lipid-staining band corresponding to beta lipoprotein normally is either associated with beta-globulin or is in an area between beta and alpha-2 globulin. This pattern was found in all the unincubated sera and in the phosphatide-free controls. However, in all of the phosphatide-containing incubated sera, both normal and hyperlipemic, the lipoprotein associated with beta-globulin migrated to the region between α-2 and α-1 globulin; *i.e.*, there was an increase in the migration velocity of beta-lipoprotein. (Fig. 1 and 2). In addition, in 5 of the 6 normal sera and 1 of the 5 hyperlipemic sera there was a definite but lesser increase in the migration of the lipid-staining band associated with α-1 globulin and albumin (alpha-lipoprotein) to an area on the strip immediately beyond albumin, after incubation with whole phosphatide complex.

2. *Exp. B.* The alcohol-soluble fraction produced little or no change in the serum lipoproteins after incubation. However, in all sera tested, incubation with the alcohol-insoluble fraction produced an increase in migration velocity similar to that demonstrated with the whole soybean phosphatide complex (Fig. 3).

3. *Exp. C.* Incubating sera with either the alcohol-soluble fraction or alcohol-insoluble fraction was associated with an increase in free fatty acid concentration, (Table I). However, no consistent differences between the 2 fractions on liberation of free fatty acids

were observed.

Discussion. In our previous unpublished preliminary investigation the same technic here described was utilized to test the effect of a fat emulsion containing 10% sesame oil and 4.5% dextrose stabilized with lecithin, on

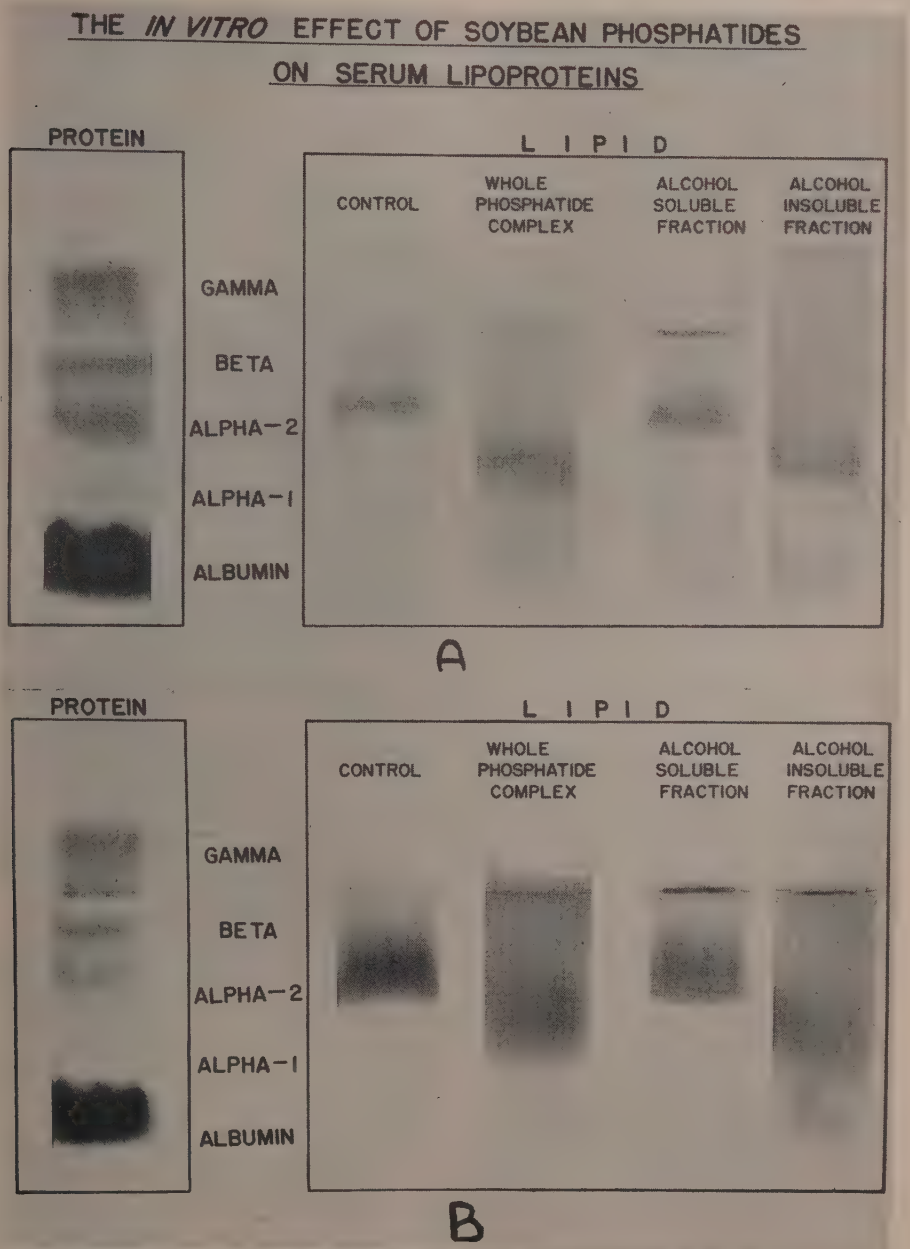

FIG. 3. Comparison of effects of incubating the whole phosphatide complex and its 2 alcohol fractions on serum from (A) a normolipemic subject and (B) a hyperlipemic subject.

TABLE I. *In Vitro* Effect of Soybean Phosphatide Fractions on Serum Free Fatty Acids.

	Free fatty acids/ml of .02 N NaOH		
	Incubated serum		
Serum	+ Alcohol-soluble fraction	+ Alcohol insoluble fraction	Untreated control
A	.16	.12	.12
B	.20	.27	.12
C*	.22	.27	.11
D	.14	.13	.05
E	.13	.17	.07
F	.13	.13	.07
G*	.10	.12	0

* Hyperlipemic.

serum lipids and lipoproteins. The results were the same as the present observations with soybean phosphatide. As this paper was being prepared for publication, the lipid emulsion effect was confirmed *in vivo*(8). The observations on the effect of the lipid emulsion and the phosphatides are of particular interest because of the recent report by Lever and Waddell(9) that serum cholesterol falls following the intravenous administration of a fat emulsion, composed of 10% cottonseed oil and 1% of the same soybean phosphatide used in the present experiments. This preparation is the natural phosphatide complex, derived from soybean and consisting of 95% phosphatide with lecithin, cephalin, and lipositol present in approximately equal concentrations. It has been demonstrated that feeding soy lecithin produces significant falls in serum cholesterol levels of man(1,11,12), of cholesterol-fed rabbits(2) and, if soy sterols are also fed, of chicks(10). However, the effects of soybean phosphatides on serum lipoproteins have not been reported. The present comparison of the effects of the 2 fractions of the whole soybean phosphatide complex suggests that lipositol is probably the compound responsible for producing the lipoprotein shift. The alcohol-soluble fraction, (consisting of approximately ⅔ lecithin and ⅓ cephalin), produced little or no change in the serum lipoproteins after incubation. In contrast, the alcohol-insoluble fraction (consisting of ⅔ lipositol and ⅓ cephalin) produced a marked increase in migration velocity similar to that demonstrated with the whole soybean phosphatide complex. Simple addition of the whole phosphatide complex or either fraction, without incubation, produced little or no increase in the migration velocity. Lipositol is a lipid of unknown structural formula, which, on acid hydrolysis, yields inositol, phosphoric acid, oleic acid, saturated fatty acids, ethanolamine and tartaric acid. Alkaline hydrolysis of this inositol phosphatide liberates an amino-reducing carbohydrate, from which galactose may be obtained after mild hydrolysis(13). These effects produced by soybean phosphatide on the migration rates of serum lipoproteins are similar to those produced *in vivo* by heparin(14) or *in vitro* by "clearing factor"(15). *In vivo* heparinized plasma has been reported to cause a release of fatty acids from beta lipoproteins(16). Others(15,17) have found that fatty acids increase the electrophoretic mobility of alpha and beta lipoproteins *in vitro*. Therefore, soybean phosphatide might be increasing the migration velocities of lipoproteins by a similar mechanism; *i.e.*, by liberating fatty acids, too. However, the equivalent effects on free fatty acids following incubation with the alcohol-soluble as well as the alcohol-insoluble fractions suggest that some other mechanism must be responsible for the difference in the effects on lipoproteins produced by the two fractions.

Although several investigators(18-21) feel that an actual transformation of beta into alpha lipoprotein occurs, this position is disputed by others. Herbst *et al.* in recent studies,(22), which demonstrated that such an *in vivo* transformation does not occur, noted a pre-albumin component similar to that observed in some of the present sera and identified it as alpha lipoprotein. Sera used in their studies were obtained from subjects with either alimentary or idiopathic hyperlipemia. In the present series, the increase in migration velocities following soybean phosphatides was demonstrated on sera from fasting normolipemic as well as hyperlipemic patients.

Conclusions. Incubation of 6 normal and 5 hyperlipemic sera with a purified extract of natural soybean phosphatides produced an increase in migration velocity of beta lipoprotein, determined by paper electrophoresis. In 5 of the normal and one of the hyperlipemic

sera increases in the migration velocity of alpha lipoprotein also were observed. Incubation of 10 normal and 9 hyperlipemic sera with the alcohol-soluble fraction of the phosphatide complex (which consists of ⅔ lecithin and ⅓ cephalin) produced little or no change in the migration velocities of the serum lipoproteins. Incubation of these sera with the alcohol-insoluble fraction, (which consists of ⅔ lipositol and ⅓ cephalin) produced an increase in the migration velocities similar to that noted with the whole phosphatide complex. Therefore, lipositol may be the compound responsible for the change observed. Determination of free fatty acids after incubation with either fraction failed to disclose any differential liberation of free fatty acids by the two fractions. The mechanism by which the alcohol-insoluble lipositol fraction increases the migration velocity of beta lipoprotein remains unexplained.

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Method for Study of Electrocardiogram of Early Chick Embryo within the Shell. (22652)

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The electric activity of isolated heart muscle of the chick embryo as an organ, or as a portion in tissue culture, has been studied by a number of investigators(1,2,4,5,6,7,10,11). Lagen and Sampson(8) employed this preparation, and Friedman and Bine(3) dissected the embryonic duck heart to study electrocardiographic changes in evaluating drugs. Today, isolated muscle from a number of animals is used in physiologic and pharmacologic studies for standardization of drug action upon the myocardium. All these technics involve considerable trauma and the use of artificial

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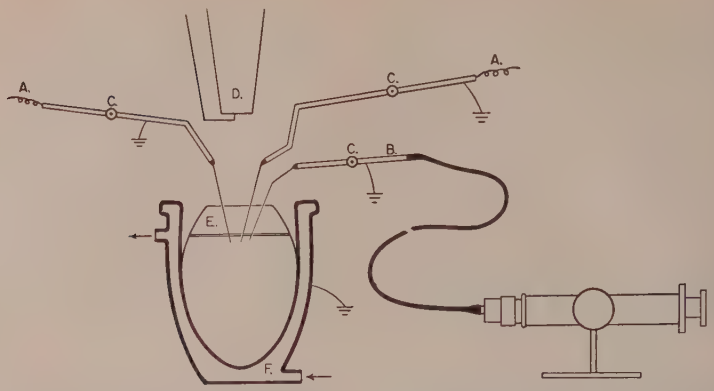

FIG. 1. Schematic drawing of the equipment. A—Connection from microelectrodes to recording apparatus; B—Insulated needle holder with ground connection; C—Universal ball type joint for needle holder; D—Dissecting microscope objectives; E—Egg air chamber; F—Egg holder with water jacket.

media with loss of spatial orientation of the fibers.

It is the purpose of this paper to describe a simple technic for obtaining electrocardiograms of early chick embryos without removal from the shell. Observations on the effect of drugs or of changes in temperature in such preparations can easily be made. The entire embryo remains in its natural environment, thus more physiological recordings can be expected.

Method. Medium sized Rhode Island eggs were incubated from 3 to 18 days at 103°F with daily rotations. From these, well developed embryos 3 to 16 days old were chosen. The eggs were mounted, air chamber on top, in a metal egg holder with a circulating water jacket (Fig. 1). The shell overlying the air sac was first sterilized with a 1% benzolkalium solution‡ and then removed, creating a window about 2 cm square. The mounted egg was placed under a dissecting microscope so that the tips of the recording electrodes were under direct vision. With incident light heart action was observed through the air sac membrane. Each electrode consisted of hypodermic stainless steel needles, one inch long, gauge #26, cut off at the hub and soldered to shielded copper wire. This was mounted in a special needle holder with ball joint (Fig. 1) to facilitate application to the selected area. The needles were insulated with varnish except for 3 mm at the tip.

To obtain maximum deflection in recordings from chick embryos 5 days old, electrodes were usually applied to the right wing and to the anterior wall of the chest. In older embryos, satisfactory recordings were obtained from the right wing, the left thigh or left wing. The needles were inserted deeply into the muscle and left in position during the period of observation.

When the embryos became quite active, as they are after 8 days of incubation, movement that interfered with the recordings was controlled by injection of 3:1000 mg curare or similar drugs.

Recordings were made on a Grass III D electroencephalograph by using EKG filter setting. The gain was adjusted to obtain a tracing of at least 7 mm deflection for the R wave. Following each recording, the instrument was calibrated with a standard value for the gain setting used. The recorder paper speed was 30 or 60 mm per second. When control tracings had been made, the drug to be tested was injected into an allantoic vein with a Pyrex glass microneedle, made in a De Fonbrune microforge and connected to an 0.01 cc tuberculin syringe. Electrocardiographic changes were recorded at various intervals. Throughout each test, a constant or

‡ Winthrop Laboratories, 1450 Broadway, New York.

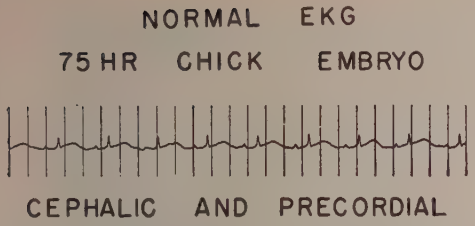

FIG. 2. R wave standard: 40 μvolts. Distance between vertical lines: 1/10 sec.

a variable temperature was maintained by means of the circulating water jacket to study temperature influence on heart action. All drug solutions were preheated before injection to the same temperature as that of the water jacket.

Results. Examples of the recordings obtained by this method from embryos at a constant temperature of 103°F are shown in Fig. 2, 3, and 4. Fig. 2 represents electrocardiograms of a chicken embryo 3 days old. At this age the P wave, the QRS complex and T wave are differentiated. The control tracing had a pulse rate of 240 beats per minute. Fig. 3 illustrates the control tracing in a 6-day-old chick embryo, and the same preparation 5 minutes after an injection of 0.1 cc of 1:10000 Ouabain (2.5 mg/cc) with heart block. Fig. 4 shows the control tracing from a 16-day-old chicken embryo. The control heart rate was 345 beats per minute. Sixty seconds after the injection of 0.2 cc of 1:1 billion Isuprel, the rate was 400 beats per minute.

Discussion. By maintaining the embryo in its natural environment, physiological conditions are maintained during recording of the EKG. The dissecting microscope permits correlation of direct heart observation with EKG changes. According to Lagen and Sampson(8) there is no nerve supply to the

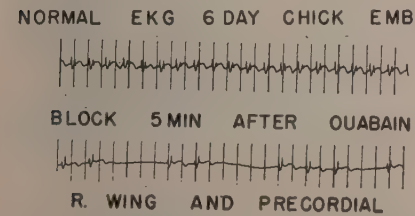

FIG. 3. R waves standard: 40 μvolts. Distance between vertical lines: 1/5 sec.

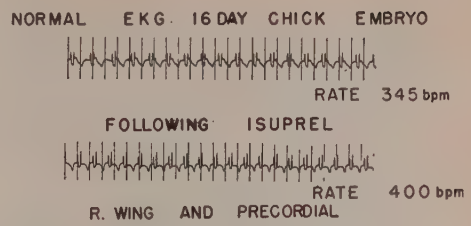

FIG. 4. R wave standard: 100 μvolts. Distance between vertical lines: 6/10 sec.

heart of the chicken embryo until the 6th day. Therefore, 3- and 4-day-old embryos provide a denervated heart preparation which may be valuable in the study of drug action when it is desirable to differentiate direct effect on the heart muscle. The use of chicken embryos within the shell to assay drugs presents definite advantages over methods currently employed, such as low cost, easy preparation, uniformity in quality, and the possibility of evaluating minute quantities of cardiac drugs. This testing procedure is also a suitable device for studying the effects of temperature changes upon the heart, and the action of drugs under hypothermia, since the time required for cooling and rewarming the entire chicken embryo is considerably less than that needed for larger animals.

Summary. A method for the recording of electrocardiograms of the chick embryo within the shell after 3 to 16 days of incubation is described. Early results on evaluation of drug action upon the myocardium at different temperatures suggest, because of the simplicity and low cost, considerable advantages over present technics.

We wish to express our acknowledgement to Dr. J. S. Lieberman who contributed to the design of the electronic equipment.

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Some Observations on Nature of Refractoriness to Histamine Liberators.* (22653)

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The ability of a variety of agents including compound 48/80† to liberate histamine *in vivo* and *in vitro* has been well established. A phenomenon of acute tolerance development has been described for many of these agents and its mechanism is commonly ascribed to depletion of available body histamine stores by previous administration of the histamine liberator(1). The experiments here reported suggest the existence of an alternate mechanism of production of a refractory state to these histamine liberators.

Methods. Mongrel dogs were anesthetized with 33 mg/kg sodium pentobarbital. Arterial blood pressure was recorded from a common carotid artery and injections were made into a femoral vein. Serial blood samples were withdrawn in heparinized syringes from the other femoral vein in those experiments in which estimations of plasma histamine were made. Plasma histamine levels were determined by direct addition of the plasma to segments of guinea pig ileum suspended in atropinized Tyrode's solution, the contraction height being compared with those produced by known amounts of histamine. Pharmacologic identification of the stimulating agent as histamine was established by the inhibitory effect of Benadryl (final concentration $1:1x10^6$).

Two methods of administration of compound 48/80 were employed: (A) 200 μg/kg compound 48/80 was administered as a single rapid injection. (B) A series of graded doses beginning with 1.5 μg/kg and increasing to 200 μg/kg compound 48/80 was administered by slow injections spaced 10 to 15 minutes apart (cumulative dose of 596 μg/kg over a 3- to 5-hour period).

Dogs thus treated were challenged with 200 μg/kg compound 48/80 injected rapidly, and in several instances by 0.1 ml/kg of 10% Tween 20.

Results. The precipitous and prolonged hypotensive effect of a rapid injection of 200 μg/kg compound 48/80 in the anesthetized dog (Fig. 1) qualitatively agrees with that

TABLE I. Plasma Histamine Concentrations before and after Administration of Compound 48/80.

Pre-treatment	No. of dogs	Plasma histamine conc. (μg/ml) Control	Min. after 200 μg per kg compound 48/80: 5	15-30
None	3	.13 ± .04*	.57 ± .10	.40 ± .05
596 g/kg, compound 48/80 †‡	4	Not detectable§	Not detectable	—

* Mean ± stand. dev.
† This amount of compound 48/80 was administered over a period of 3-5 hr in a series of graded doses ranging from 1.5-200 μg/kg.
‡ No histamine was detectable in plasma samples obtained every 30 min. during this period.
§ Sensitivity of the histamine assay method is estimated to be .05 μg histamine.

* This investigation was supported by research grant G-4077 (C) from the National Institutes of Health, Public Health Service.

† Compound 48/80 is a mixture of the dimers, trimers, and tetramers resulting from the condensation of N-methylhomoanisylamine and formaldehyde. Compound 48/80 was kindly made available to us by Dr. E. J. de Beer, The Wellcome Research Laboratories, Tuckahoe, N. Y.

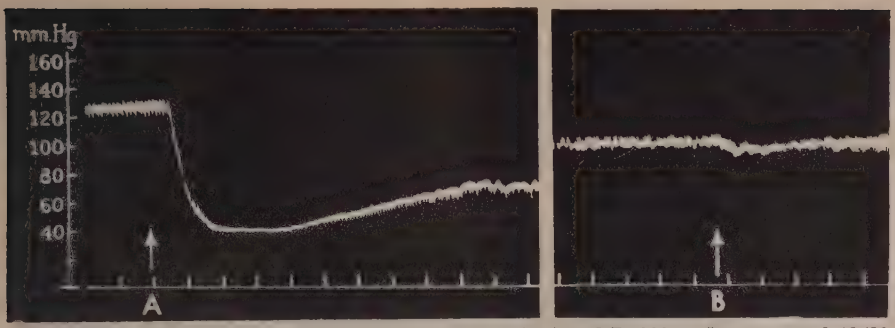

FIG. 1. Effect of compound 48/80 on blood pressure: At A and B, 200 μg/kg compound 48/80 was inj. rapidly. Base line signal interval, 1 min.; interval between sections, 69 min.

reported by others(2). An increase in plasma histamine concentration was found to occur in the early period of hypotension (Table I).

When the administration of compound 48/80 was modified to a series of graded doses ranging from 1.5 to 200 μg/kg, it was possible to inject a total of 596 μg/kg over 3 to 5 hours without producing changes in the blood pressure; these animals were subsequently refractory to the rapid injection of 200 μg/kg (Fig. 2). No changes in plasma histamine concentration were detectable during the period of administration of the graded doses or following the rapid injection of 200 μg/kg compound 48/80 in these animals (Table I).

Tween 20 produced a marked hypotensive effect in both groups of 48/80 refractory animals.

Discussion. The development of tolerance to the action of histamine liberators has been described in a variety of species. Feldberg and Paton(3) demonstrated the ability of various histamine liberators to deplete tissues of virtually all of their available histamine.

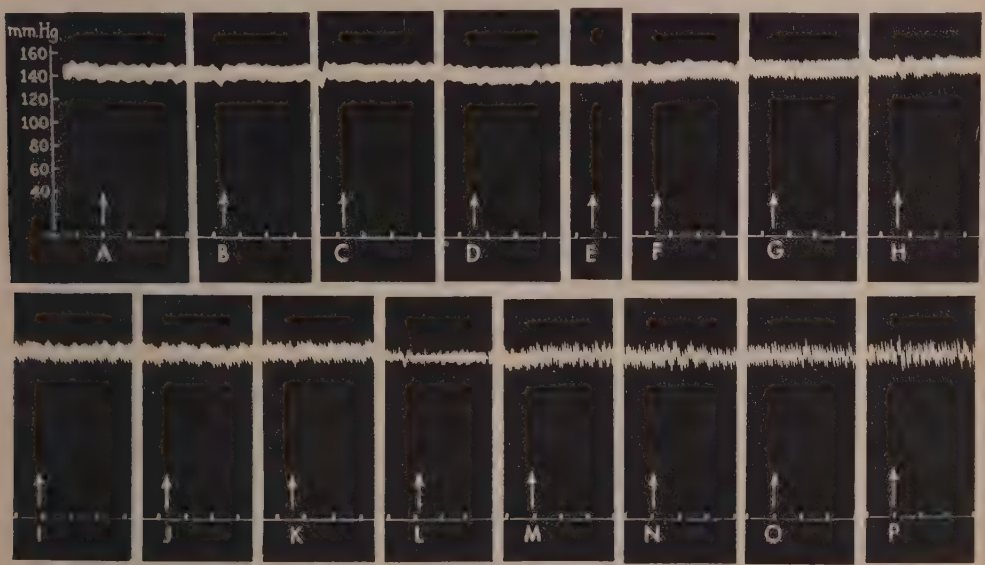

FIG. 2. Effect of compound 48/80 on blood pressure: Compound 48/80 was administered in a graded dosage schedule by slow injections as follows: At A and B, 1.5; C and D, 3.1; E and F, 6.3; G and H, 12.5; I and J, 25; K and L, 50; M and N, 100; O, 200 μg/kg. At P, 200 μg/kg was inj. rapidly. Base line signal interval, 1 min.; interval between successive injections, 5-10 min.

Sufficient histamine is released by compound 48/80 in the cat and dog to account for its hypotensive action, and refractoriness to this compound is associated with its inability to liberate more histamine(4). The experiments of Feldberg and Talesnik(1) indicate that refractoriness to compound 48/80 is associated with the depletion of available histamine body stores by prior administration of the histamine liberator. Mayeda(5) has demonstrated cross-tolerance to the histamine liberating action of peptone and sinomeminine, and Slomka and Goth(6) have demonstrated a similar cross-tolerance between compound 48/80 and several morphinan compounds. This cross-tolerance may also be explained on the basis of depletion of available histamine body stores. Indeed, the marked reduction in the edema produced by light in porphyrin-treated rats whose skin histamine had been markedly depleted by prior administration of compound 48/80(1) lends support to this hypothesis. Although depletion of available histamine stores provides an obvious mechanism for the development of tolerance to histamine liberators, the results of our experiments suggest that an alternate mechanism of inducing a refractory state exists. Since no increase in circulating histamine concentrations was detectable during the development of the refractory state to compound 48/80 with a graded dosage schedule, it appears unlikely that a significant amount of histamine was liberated, *i.e.* enough to explain the refractory state by depletion of the available histamine body stores. The responsiveness of the cardiovascular systems of such 48/80 refractory dogs to exogenous histamine is unaltered. The ability of the histamine liberator Tween 20 to produce its characteristic hypotensive effects in 48/80 refractory animals further suggests that the depletion of available histamine body stores has not been accomplished. The ability of compound 48/80(7) and Tween 20(8) to liberate histamine from liver mitochondria has been demonstrated.

Although its mechanism has not been elucidated, an interpretation of our results suggest that acute refractoriness to the histamine liberator compound 48/80 has been developed in dogs by means other than that of depletion of available body stores of histamine.

Summary. The administration of a series of graded doses of compound 48/80 produces a refractory state to the histamine liberating action of this compound. This refractory state appears to be effected by a means other than that of depletion of available histamine body stores.

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Tissue Distribution of Parenteral Co^{60} Vitamin B_{12} in Mouse, Hamster, Rat and Guinea Pig.* (22654)

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(Introduced by B. S. Walker.)
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Species differences in the tissue distribution of parenterally administered Co^{60} vit B_{12} ($Co^{60}B_{12}$) have been suggested by recent studies. In the rat, the kidneys were found to be the main site of $Co^{60}B_{12}$ localization even at a time when urinary excretion was absent(1,2,3). In man, the greatest percentage of a parenterally injected dose of $Co^{60}B_{12}$ was found in the liver(4). In the chick(5) minimal localization of $Co^{60}B_{12}$ was found in the liver and kidney. In this study parenteral $Co^{60}B_{12}$ was administered to hamsters, rats, mice and guinea pigs in order to elaborate on possible species differences in its tissue distribution.

Methods. Experimental plan: Eight adult male Swiss white mice weighing 25-40 g, 8 adult hamsters (4 male and 4 female) weighing 85-120 g, 7 adult male Sprague-Dawley rats weighing 200-230 g, and 4 adult male guinea pigs weighing 450-600 g, were subcutaneously injected under ether anesthesia with $Co^{60}B_{12}$ (secured from Merck and Co., Rahway, N.J.). The dose was contained in a volume of 1.0 ml, except for mice, where 0.5 ml was given. The dose injected ranged from 0.10 to 0.40 μc of Co^{60}. Mice received 0.15 μg of B_{12}, hamsters 0.70 μg, rats 0.30 μg and guinea pigs 0.26 μg. After injection, rats and guinea pigs were housed in metabolic cages so as to allow separate urine and stool collections. Hamsters were housed in wire cages under which 2 thicknesses of Whatman filter paper #3 were spread. All stools fell on the filter paper while the urine was absorbed into the paper. Mice were housed in 2,000 ml beakers. The bottoms of the beakers were also lined with this filter paper on which urine and stools were deposited. Four days after the injection of the Co^{60} B_{12}, animals were anesthetized, exsanguinated by cardiac puncture, and the appropriate organs removed.

Preparation of specimens for analysis: (a) *Organs.* The liver, kidneys, spleen, heart, testes and portions of the hind leg musculature were removed and weighed. Organs were placed into flasks containing 10 ml of 2N NaOH, except for the livers where 20 ml was used. The organs were digested in alkali at room temperature for 24 hours. Then, if necessary, gentle heating for 30 to 60 seconds brought the gel-like digest into a liquid state. Measurements of radioactivity were made on duplicate 4 ml aliquots of these tissue digests. The total volume of the tissue digest solution was assumed to be equal to 10 ml when organ weight was less than 0.75 g as the volume contributed by the dissolved organ was negligible. For heavier organs the total volume of the digest was measured in a 25 ml graduated cylinder. (b) *Urine, stools, and blood.* Independent total urine collections were secured for the first 24 hours and for the following 3 days for the rats and guinea pigs. The total urine volume was measured in a 50 ml graduated cylinder. Radioactivity was determined on 4 ml aliquots of urine. For hamsters and mice, the filter paper on which the urine had dried was cut into small pieces and placed into 1¾ by 3⅛ inch glass bottles in which radioactivity was measured. The radioactivity in 4-day stool collections was also determined in 1¾ by 3¼ inch glass bottles. One to 4 ml of oxalated blood (hamsters and rats were only done) was used for radioactivity assay. (c) *Carcasses.* Mice and hamster carcasses were placed into 1¾ by 3¼ inch glass bottles while the rat carcasses were placed into 2½ by 6 inch glass bottles. Car-

* This work was supported in part by grants from the United States Public Health Service and the Atomic Energy Commission.

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TABLE I. Tissue Distribution and Excretion of $Co^{60}B_{12}$ in Mice, Rats, Hamsters and Guinea Pigs.

	Mice		Rats		Hamsters		Guinea pigs	
Organ	% of inj. dose*	% of dose* g wet wt	% of inj. dose	% of dose g wet wt	% of inj. dose	% of dose g wet wt	% of inj. dose	% of dose g wet wt
Kidney	6.2 ± .4	13.0 ± 1.4	16.2 ± 2.9	9.7 ± 1.9	7.2 ± .6	6.3 ± .1	2.4 ± .1	.3 ± 0
Liver	23.7 ± 2.5	11.2 ± 1.1	7.4 ± .4	.8 ± 0	18.0 ± 2.9	6.1 ± .1	29.7 ± 4.1	1.3 ± 0
Testes	1.5 ± .1	7.0 ± 1.1	1.0 ± 0	.6 ± 0	not done	—	.3 ± 0	.1 ± 0
Spleen	1.4 ± .2	1.7 ± .5	.8 ± 0	.6 ± 0	.3 ± 0	.8 ± 0	.2 ± 0	.3 ± 0
Heart	.4 ± .1	1.8 ± .1	.7 ± 0	.9 ± 0	.4 ± 0	.6 ± 0	.2 ± 0	.1 ± 0
Muscle	15.9 ± .9	1.3 ± 0	13.6 ± 1.1	.2 ± 0	7.7 ± 1.5	.3 ± 0	not done	—
Carcass	26.0 ± 2.9	—	34.5 ± 4.0	—	43.0 ± 3.8	—	" "	—
Urine	5.9 ± 1.8	—	7.4 ± 1.1	—	14.1 ± 1.0	—	12.5 ± .9	—
Stool	15.5 ± 1.8	—	18.5 ± 6.2	—	3.1 ± .9	—	5.5 ± .1	—
Recovery	106.1		100.7		95.1		50.8	

* Mean and stand. error of mean.

cass radioactivity measurements were made in these bottles. Because of their large size, the guinea pig carcasses could not be counted.

Radioactivity determination: A 1:100 dilution of the injected $Co^{60}B_{12}$ was made and varying aliquots were then used as standards for the varying conditions employed in the counting of organ digests, excreta and carcasses. By adding water as needed, the height of the standard in the glass bottles was always the same as that of the specimen being counted *i.e.*, stools, urine or carcasses. Blood, tissue and liquid urine samples were counted in a scintillation well-type counter (thallium activated sodium iodide scintillation crystal). The radioactivity of the organ samples usually counted 3-12 times background, except for the muscle, heart, blood and spleen which usually counted 1-3 times background. Samples were counted long enough to give a counting error of less than 2%, except for the slow-counting samples where a 5-8% counting accuracy was obtained. Duplicate samples usually agreed within 1-5%. All samples contained in the 1¼ by 3¼ inch glass bottles were counted by placing them on top of the above-mentioned sodium iodide well crystal. The diameter of these was just about equal to that of the diameter of the well. Under similar geometry, a standard was also counted. The 6 inch bottles containing the rat carcasses were marked into 3 equal 2 inch segments. With the bottle in the horizontal position, and directly in contact with a solid 1 by 1 inch thallium activated sodium iodide crystal, each 2 inch segment of the bottle containing the carcass was counted. Under similar geometry the bottle containing the standard was counted.

Calculations. (a) The percent radioactivity in organ =

$$\frac{\text{cpm per ml organ digest} \times \text{total volume digest}}{\text{cpm injected}} \times 100$$

In calculating the total percent uptake in the muscle mass, 40% of the animal's weight was assumed to be muscle. For the total percent uptake in blood, a blood volume was estimated based on the figure of 69 ml/kg body weight.

Results. The organ distribution and excretion of a parenteral dose of $Co^{60}B_{12}$ in the rat, guinea pig, mouse and hamster are shown in Table I.

Discussion. In the rat the tissue distribution of a parenterally injected dose of $Co^{60}B_{12}$ was similar to that found by Rosenblum *et al.* (1). Similar studies on the mouse, hamster and guinea pig are not available in the literature. Differences in the major organ sites of $Co^{60}B_{12}$ localization were noted in the 4 species studied. In the hamster, guinea pig and mouse, the liver was the chief organ of localization whereas in the rat the kidney was the chief site. The high $Co^{60}B_{12}$ uptake of the rat kidney is probably unrelated to its known role in the excretion of large doses of parenterally injected vit B_{12}(3,6,7), since the dose given the rat was comparable to that given the other 3 species. Furthermore, the other 3 species studied had comparable amounts of

radioactivity excreted in the urine without an increase in kidney $Co^{60}B_{12}$ uptake. At the time the high rat renal $Co^{60}B_{12}$ uptake was found, urinary excretion of the radiovitamin was negligible making it highly unlikely that the $Co^{60}B_{12}$ in the kidneys was there to be excreted. This is further supported by the observations of Harte(2), who found that high renal $Co^{60}B_{12}$ levels were maintained for as long as 90 days after injection despite the absence of urinary excretion after the first day. It would thus appear that the kidney represents an important site of $Co^{60}B_{12}$ localization in the rat rather than a mere storage site for further urinary excretion. The high $Co^{60}B_{12}$ concentration in the liver of the mouse, hamster and guinea pig is comparable to that found in man after the injection of labelled vit B_{12}(4). The rat, with the chief localization of injected $Co^{60}B_{12}$ in the kidney, is not analogous to man. It would, therefore, appear hazardous to relate to man the results of the experiments in the rat.

Differences in the routes of $Co^{60}B_{12}$ excretion were noted in the 4 species studied despite the administration of comparable amounts of vit B_{12} in relation to body weight. The stools were the chief routes of excretion in the rat and mouse, whereas the urine was the chief excretory pathway in the hamster and guinea pig. In previous studies in the rat (1,8) the urine was the major excretory pathway containing 50-90% of a parenteral dose of $Co^{60}B_{12}$. These results differ markedly from those found in this study and are probably due to the large doses of vit B_{12} (4-20 μg) used in these studies. There is no available data on the excretion of parenteral $Co^{60}B_{12}$ in the other 3 species studied. The present investigation suggests that the stools may be an important route for the excretion of vit B_{12} in the rat and mouse. Radioactivity as measured in this study merely indicates the presence of the Co^{60} moiety of the vit B_{12} molecule. Thus, the radioactivity may be due to vit B_{12} or to an intermediate degradation product of the vitamin. After parenteral administration of $Co^{60}B_{12}$ to rats, the radioactivity excreted in the urine was found to be due to vit B_{12}, whereas that in the stool was unassociated with vit B_{12} activity(1). The radioactivity in the kidneys was found to be due to vit B_{12} in a similar study(8). It appears likely that the radioactivity in the kidneys in this study is due to vit B_{12}. However, concerning the other organs, this question cannot be answered.

Summary. The excretion and tissue distribution of parenterally administered $Co^{60}B_{12}$ has been studied in the rat, hamster, mouse and guinea pig. The liver contained more radioactivity than any other organ in the hamster, mouse and guinea pig, whereas the kidneys contained more radioactivity than any other organ in the rat. In the rat and mouse, more radioactivity was excreted in the stools than in the urine whereas in the guinea pig and hamster, the urine was the chief excretory pathway.

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Multiple Intracellular Recording from Atrial and Sino-atrial Cells: Correlation with Contractile Tension.* (22655)

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(Introduced by T. A. Loomis.)
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In the course of microelectrode studies of rabbit atria in this laboratory it was noted that cells in the region of the sino-atrial node failed to respond to as high frequencies of electrical stimulation as did atrial cells. The present study was undertaken to investigate this phenomenon by means of simultaneous recording of atrial and sino-atrial cell action potentials. An effort was made to correlate the contractile response with the electrical activity of atrial muscle.

Methods. Virgin female albino rabbits were sacrificed by a blow on the neck and quickly exsanguinated. The thorax was opened and the entire heart quickly removed and dropped into oxygenated Tyrode solution, as modified by Hoffman and Suckling(1). In this solution, through which oxygen (95%)-carbon dioxide (5%) constantly bubbled, the combined atria were carefully separated from the ventricles and then mounted in a Plexiglas bath containing Tyrode solution. The bath was oxygenated through a diffusion dish which was incorporated into the chamber. The nutrient solution constantly flowed through at a rate of 3 cc per minute. The right atrial appendage was fastened to 2 silver hooks which also served as the stimulating electrode. The connective tissue in the septal area was secured to the arm of a sensitive strain gauge (Grass force displacement transducer), and the left atrium was allowed to float freely in the chamber. Stimulation was applied by means of a Grass Model 3C Stimulator. The output was passed through an isolation transformer to reduce the shock artifact. The signal produced by distortion of the strain gauge arm during contraction, was fed into a preamplifier, which in turn supplied the input to a DC amplifier of a Sanborn 4 channel recorder. Transmembrane potentials were determined with two cathode follower circuit units(2), using simultaneously 2 glass capillary electrodes with tip diameters of 0.5 μ or less. One electrode was used in the atrial myocardium exclusive of pacemaker region, while the other was used to explore the pacemaker area. Each of these circuits was connected to a separate DC amplifier in a Sanborn 4 channel recorder. The temperature of the bath was maintained between 28-30°C in order to reduce the spontaneous rate, lengthen the duration of the action potential and to maintain better contractility.

Results. Tracings of typical records obtained during simultaneous impalement of atrial and sino-atrial cells are seen in Fig. 1. The dotted line represents the zero potential level. Sino-atrial cell firing can be recognized by the slow diastolic prepotential development as noted in the upper portion of the tracings. After a control period, electrical stimulation was initiated and maintained for 1 to 3 seconds. Recovery represents the beginning of spontaneous activity following electrical stimulation. At the 2 frequencies depicted (3.75 and 4.6 stimuli per second), the atrial cell follows, while the sino-atrial cell follows at the lower but not at the higher frequency. In general, the amplitude of atrial cell action potentials was 90-120 mv, while that of sino-atrial cells was lower (50 to 70 mv). The lower values for sino-atrial cell action potentials are in agreement with the reports of other investigators working with rabbit atria(2) and frog sinus venosus(3,4).

Fig. 2 summarizes the results obtained on frequency of response of atrial and sino-atrial cells versus frequency of electrical stimulation applied. Blackened circles (sino-atrial responses) and open circles (atrial cell responses) represent the arithmetic mean of 2

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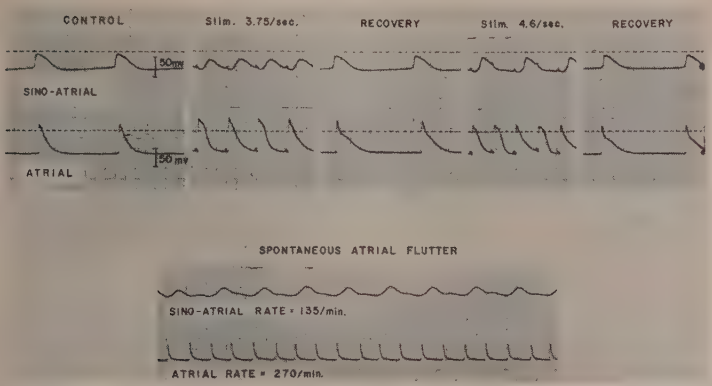

FIG. 1. Simultaneous recording of sino-atrial and atrial cell action potentials during electrical stimulation and spontaneous atrial flutter. Dotted line represents zero potential level. Each small division represents one mm. Paper speed, 50 mm/sec.

to 18 determinations. At lower frequencies the minimum number of determinations was 4 to 5, with a maximum at 18, while at higher frequencies (8-16 stimuli per second) a minimum of 2 determinations were made. The vertical bars are ranges of responses at any one frequency. Inspection of the graph discloses a linear relationship (1:1) for atrial cells at stimulation frequencies as high as 10 per second. The slope of the line drawn through the open circles is unity. Sino-atrial cells fail to follow rates as low as 4 per second. Stimulation frequencies beyond this point resulted in an integral ratio of atrial to sino-atrial cell responses; being 2:1 up to a frequency of 7.5 per second, then 3:1 and 4:1 at higher frequencies.

A recording taken at a time of spontaneous atrial flutter is shown in Fig. 1. There is a 2:1 ratio between atrial and sino-atrial cell firing, a phenomenon which parallels the results obtained from electrical stimulation.

Shown in Fig. 3 are simultaneous recordings of action potentials of an atrial cell and the tension developed by the right atrium. At the onset of electrical stimulation there is a decrease in tension (3rd beat, Fig. 3). This has been reported for isolated papillary muscle by Gold and Cattell(5). As stimulation was continued, the tension alternated in a regular manner such that a beat in which high tension developed was followed by a beat of low tension. Both the high and low tension contractions exhibited a positive staircase effect(6,7) during the applied electrical stimulus. When stimulation was stopped, there was a marked increase in tension on recovery with the occurrence of a negative staircase effect. It is of interest to note the repolarization phase of atrial cell action potentials during electrical stimulation (4th to 6th and 9th to the 13th beats in Fig. 1, 3rd to the 12th beats in Fig. 3). Alternation in repolarization configuration can be seen; every other beat exhibited an exponential repolarization in contrast to the more sigmoid repolarization of the intervening beats. In Fig. 3, alternation becomes apparent during continued electrical stimulation. The tension is greater in those cycles where repolarization

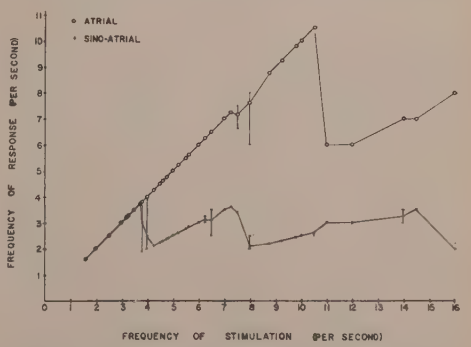

FIG. 2. Graph summarizing data of simultaneous recording of atrial and sino-atrial cell action potentials during electrical stimulation. Open circles, atrial cell responses; blackened circles, sino-atrial cell responses. Ordinate: frequency of electrical stimulation. Abscissa: frequency of response.

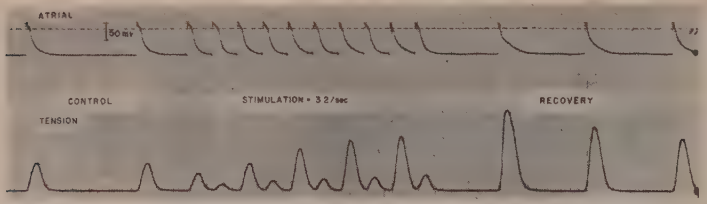

FIG. 3. Simultaneous recording of atrial cell action potentials (upper tracing) and tension developed by right atrium (lower tracing). Dotted line represents zero potential level. Each small division represents one mm. Paper speed, 50 mm/sec.

was exponential rather than sigmoid (5th, 7th, 9th and 11th beats in Fig. 3). Post-stimulation potentiation of tension is accompanied by a repolarization curve which is considerably longer in duration than those of the control action potentials or those occurring during electrical stimulation (7th and 15th beats in Fig. 1, 13th beat in Fig. 3). Tension development and action potential configuration returned to control values after 4 or 5 spontaneous beats.

Discussion. The difference in the follow rates of sino-atrial and atrial cells is to be expected on the basis of the much longer duration of the action potentials of sino-atrial cells(2,8,9). An electrical stimulus falling during the earlier phases of repolarization fails to excite sino-atrial cells, while on the same time scale the stimulus would excite atrial cells which have already returned to the polarized state. The sino-atrial region appears to act as pacemaker normally because it is usually in a partially depolarized state (2,8,9) and has the greatest tendency to undergo cyclic spontaneous depolarization. Certainly, pacemaker activity cannot be explained on the basis of refractory period, since the experiments reported here indicate a longer refractory period for sino-atrial cells. The same discrepancy between atrial and sino-atrial cells has been observed when a relatively rapid "flutter" has been induced in the preparation. When the rate of stimulation exceeded that rate at which the sino-atrial cells could follow, it was observed that alternation in both electrical and mechanical phenomena occurred. That is, the repolarization of atrial cells alternated between a sigmoid and exponential curve. At the same time, total contractile tension alternated in magnitude. It was observed that high tension was associated with the exponential type repolarization curves whereas low tension matched the sigmoid type repolarization. Inspection of simultaneous tracings of atrial and sino-atrial spikes showed that effective depolarization in sino-atrial cells also was coincidental with the exponential repolarization curve in atrial cells. In those experiments in which simultaneous atrial and sino-atrial electrical events and contractile tension all were observed, the same correlation was observed within limits. The cause-effect relationship is not yet clear. However, at higher frequencies a 4:1 ratio between atrial and sino-atrial response may be evident without a similar pattern in tension magnitude. It is felt that the correlation between sino-atrial cell firing and tension, when tension is alternating at lower frequencies, probably is coincidental. On the other hand, the relationship between atrial cell repolarization and tension, which is a consistent finding in this preparation, appears to be valid at all frequencies, although they are not related in any simple way. Further investigation of this phenomenon is in progress.

Post-stimulation potentiation and post-extrasystolic potentiation in the heart have been studied by other investigators. The studies of Rosin and Farah(10) were conducted on isolated rabbit atria and ventricles; those of Furchgott and Sleator(11) on guinea pig atria. Changes in contractility were qualitatively similar to those reported here. Neither of these authors attempted to relate contractility to transmembrane potentials. This has been done recently by Hoffman, Bindler and Suckling(12) using cat papillary muscle. However, the post-extra-

systolic potentiation observed by the latter investigators was not accompanied by a significant change in membrane repolarization.

Summary. 1. Cells in the region of the sino-atrial node of rabbit are unable to respond to as high a frequency of stimulation as those cells not included in the pacemaker area. 2. The same phenomenon has been observed during rapid atrial arrhythmias in the absence of electrical stimulation. 3. The magnitude of the contractile tension developed by the atrium is related to repolarization of atrial cell action potentials, being greater for those demonstrating exponential rather than sigmoid curves. This is a consistent finding occurring during periods of electrical stimulation and with the first few spontaneous beats following stimulation, but the tension and action potential repolarization are not related to each other in any simple way.

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Effect of DPPD, Methylene Blue, BHT, and Hydroquinone on Reproductive Process in the Rat.* (22656)

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Although several compounds have been found to prevent or cure certain vit. E deficiency symptoms, such as brown discoloration of the uterus and cloudy swelling of the convoluted tubules of the kidney, only 2, diphenyl-p-phenylenediamine (DPPD) and methylene blue, have been reported to substitute for tocopherol in preventing resorption-gestation in vit. E-deficient female rats. In our laboratory also, preliminary experiments indicated that methylene blue and DPPD gave slight positive responses under the conditions of our standard vit. E bioassay procedure(1). Consequently, we were led to investigate this apparent vit. E activity further.

Methylene blue. The results of vit. E bioassays in which the responses of 5 levels of methylene blue were compared with five levels of standard, *d-α*-tocopherol. showed median fertility doses (MFD) of 36 mg and 0.29 mg respectively. Thus, methylene blue has less than 1% the activity of *α*-tocopherol. However, during the course of these experiments, Moore, Sharman, and Ward(2) reported that under the conditions of vit. E bioassay in their laboratory, methylene blue was completely negative. The bioassay procedures used at Cambridge and by us were practically identical except for length of time of depletion. Moore used 5 months and we used about 5 weeks. Consequently, the slight positive response we obtained for methylene blue may have been due to its protective or sparing effect on the small, residual quantities of *α*-tocopherol still remaining in the tissues of our animals after only 5 weeks deple-

* Communication No. 233.

TABLE I. Effect of DPPD* and *d*-α-Tocopheryl Acetate on Reproductive Performance of Vit. E-Deficient Female Rats.

	Dietary supplement		
	Negative control, no suppl.	0.2% DPPD	0.002% *d*-α-tocopheryl acetate
Fertility index (No. of females pregnant to No. mated)	3/3	9/9	9/9
Litter efficiency (% of pregnant animals with at least 1 viable fetus)	0	33	100
Mortality (No. of females dying at parturition)		2	0
Mean length of gestation (days)		25	22
Mean litter size		2.3	7.8
Viability index (No. of young alive at 3 days to No. born)		0/7	63/70
Lactation index (No. of young weaned to No. alive at 3 days)		—	61/63

* Feed grade DPPD.

tion. Quite recently, Christensen, Dam, and Gortner(3) revised their original conclusion (4) since methylene blue has been found to be ineffective as vit. E in preventing fetal resorption if the depletion diet is rigorously freed of tocopherol, thus eliminating the sparing effect of methylene blue on small, residual amounts of tocopherols in the vit. E-low ration. Therefore, methylene blue is not a vit. E substitute for preventing fetal resorption and, furthermore, should be used with caution, according to Moore(5), since at high levels of 0.252% in the diet it caused growth depression and severe damage (fragmentation) to the spleen of male rats. We also observed that all female rats died when supplemented with 100 mg/day of methylene blue.

DPPD. Results of a standard bioassay of DPPD in a Tween-water dispersion for vit. E potency showed that under the conditions of our test (5-12 week depletion period), DPPD possessed 7% the activity of the *d*-α-tocopherol standard on a molar basis. Johnson and Goodyear(6) reported that DPPD (0.385 mg/wk) supported reproduction in female rats maintained on a vit. E-low ration from weaning. Subsequent experiments showed(7) that this vit. E-like effect of DPPD disappeared as soon as the animals' tissue stores of tocopherol were used up.

A further test of the apparent anti-sterility potency of DPPD was conducted in which DPPD was fed mixed into the vit. E-low diet and fed to vit. E-deficient female rats beginning 7 days prior to mating. In Table I are shown the results obtained in comparison with a control group treated identically except that the diet was supplemented with 0.002% *d*-α-tocopheryl acetate instead of DPPD. DPPD in the diet (0.2%) was less effective than *d*-α-tocopheryl acetate (0.002%) in preventing typical vit. E-deficiency gestation resorptions, as evidenced by "litter efficiencies" of 33% and 100%, respectively. But more striking was the effect of DPPD on the duration of gestation and on mortality of young. Gestation was prolonged to 25 days from a normal of 22 days, and all of the newborn rats died before 3 days of age.

Other experiments with a vit. E-free diet or a stock diet confirmed these adverse effects of DPPD. Typical results are shown in Table II. Butylated hydroxytoluene (BHT) and hydroquinone (HQ) were also fed to determine whether these antioxidants would show similar toxicity in pregnant animals. Neither BHT nor HQ at the 0.3% level induced the prolonged gestation and mortality characteristic of DPPD. BHT at the 1.55% level caused a drastic weight loss in the pregnant females which was associated with fetal deaths. This response was not unexpected since at this level of feeding the rats were receiving each day about half the single dose LD_{50} of BHT as reported by Deichmann *et al.* (8).

The DPPD effect is more pronounced in animals fed the supplemented "stock diets" 10 days before insemination than in those fed the supplemented "vit. E-low diets fortified with *d*-α-tocopheryl acetate" starting on the day of insemination. Since these experiments were conducted at different times, comparisons should be made with caution. The most

TABLE II. Effect of DPPD* on Reproduction in Female Rats.

Group No.	Supplement and level in diet (%)	Fertility index	Litter efficiency	Mortality index	Mean length of gestation	Mean litter size	Viability index	Lactation index
	Vit. E-low diet† + .001% *d*-α-tocopheryl acetate (supplemented diets fed starting on day of insemination)							
1	Negative control	14/17	100	0/17	22	7.7	73/ 85	71/73
2	DPPD .0125	8/11	60	1/11	23	6.2	18/ 31	18/18
3	" .0625	11/11	88	0/11	24	5.1	8/ 51	6/ 8
4	" .313	10/11	90	2/11	25	6.0	0/ 54	
5	" 1.55	8/11	50	1/11	26	7.3	0/ 29	
6	BHT .0125	9/11	100	0/11	22	8.7	71/ 78	70/71
7	" .0625	11/11	100	0/11	22	7.5	73/ 82	59/73
8	" .313	11/11	91	0/11	22	8.7	72/ 87	69/72
9	" 1.55	9/11	0	0/11	22			
	Stock diet‡ (supplemented diets fed 10 days prior to insemination)							
1	Negative control	16/17	88	1/17	23	10.6	71/104	69/71
2	DPPD .0125	10/12	83	0/12	24	7.9	4/ 79	3/ 4
3	" .0625	15/17	70	5/17	25	4.9	0/ 49	
4	" .313	8/10	60	5/10	25	5.3	0/ 16	
5	" 1.55	11/13	33	7/13	25	4.7	0/ 14	
6	HQ .003	10/10	100	0/10	22	8.1	79/ 84	78/79
7	" .3	10/10	100	0/10	23	7.8	65/ 76	62/65

* Feed grade DPPD. † Diet #301 of Harris and Ludwig(9). ‡ Diet of Ames *et al.*(10) except with linseed meal replacing soybean meal and partially hydrogenated vegetable oil (Primex) replacing margarine oil.

sensitive criterion of DPPD feeding, a high incidence of early mortality of young, occurred in both experiments in groups fed 0.0125% DPPD, the lowest level tested. In another experiment, supplementation of the diet with 400,000 I.U./kg of vit. A or 0.1% of *d*-α-tocopheryl acetate failed to alleviate the toxicity. Those young rats which survived this early period of life (3 days) were able to suckle successfully, as evidenced by a normal lactation index. Considerable maternal mortality occurred at levels of 0.0625% DPPD and higher in the stock diet. The abnormality leading to death seemed to be largely mechanical. Gestation was prolonged, in some instances as much as 36%. Probably the fetuses continued to grow *in utero* until, when parturition did occur, the birth process was exceedingly difficult and prolonged. Many of the young were born dead and most of the mothers that died did so during parturition. Other symptoms occasionally observed were vaginal bleeding and prolapse of the uterus. The physiological defect caused by DPPD is probably failure of "the trigger mechanism" which starts the birth process at the proper time.

Summary and conclusions. 1) Vit. E. bioassays (prevention of resorption-gestation) of both methylene blue and DPPD indicated a low order of activity. However, this apparent tocopherol-like potency was due probably to a protective or sparing effect on small residual quantities of vit. E in the diet and in the body tissues. Under conditions where the diet was rigorously freed of tocopherol and the depletion period was extended, methylene blue and DPPD were found by other investigators to be completely devoid of vit. E activity. 2) At higher levels of supplementation, both methylene blue and DPPD were toxic. Methylene blue in doses of 100 mg/day killed pregnant female rats. Feeding pregnant rats diets containing DPPD at a level of 0.0125% in the diet resulted in mortality of their young. Higher levels of DPPD induced prolonged gestation and maternal mortality. Neither BHT (0.313%) nor hydroquinone (0.3%) produced these symptoms of toxicity, but BHT at the 1.55% level in the diet resulted in drastic loss of weight and fetal deaths.

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Mouse Pathogenicity with Gastric Mucin as an Index of Staphylococcus Virulence. (22657)

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Bacterial virulence, defined broadly as the capacity of an organism to invade and multiply in tissues and produce disease or death, is best measured by observing pathological effects induced by the microorganism in a suitable host. Animal virulence tests, although employed for a number of other bacterial species, have not been applied to staphylococci chiefly because of their inconstant and irregular pathogenicity for laboratory animals. Such biochemical properties as coagulase, pigment and hemolysin production as well as mannite fermentation are utilized instead with the greatest reliance being placed on the first, but the validity of these criteria as indices of virulence in all instances has been subject to serious question by many investigators. Lack and Wailling(1), as one example, in a study on the factors contributing to pathogenicity of staphylococci, state, "While there is strong evidence that the absence of coagulase production is associated with nonpathogenicity, there is no evidence that all coagulase-producing strains are pathogenic."

Hog gastric mucin has found wide application as an adjuvant in converting infections caused by organisms considered of low virulence for mice on intraperitoneal injection into rapidly lethal ones. Among the numerous bacterial species reported amenable to its action in this regard are some strains of staphylococci(2-4). Ercoli *et al.*(5) have shown that staphylococci injected intraperitoneally into mice with mucin remain viable and spread from the abdominal cavity, while without it they are rapidly destroyed. It was believed of interest to ascertain whether the mouse could be converted from a relatively resistant host to staphylococcus infection by the intraperitoneal route into one readily susceptible, by means of the adjuvant, and if so, to determine whether this procedure could be adapted to the differentiation of virulent from avirulent members in the same manner that animal inoculations are employed with corynebacteria, clostridia, mycobacteria, etc. To facilitate this evaluation, the *in vitro* presumptive biochemical reactions of a series of strains of staphylococci were compared with their intraperitoneal pathogenicity for mice with mucin.

Materials and methods. 60 strains of staphylococci recently isolated from a variety of such clinical sources as suppurative lesions, blood, nose and throat, urine and skin were employed. 5% hog gastric mucin (Type 1701-Wilson) was prepared according to Miller(6). Ten albino Swiss mice, weighing approximately 15-20 g, were used for each bacterial strain, of which 5 received 0.5 ml of 5% hog gastric mucin and the remainder 0.5 ml of physiological saline intraperi-

toneally 15-20 minutes prior to the intraperitoneal injection into each mouse of 0.25 ml of a 16 hour veal infusion broth culture. Animals were observed 48 hours and all deaths were recorded. In addition, one group of 10 mice received 0.75 ml of mucin and another group of 10 mice received 0.75 ml of veal infusion broth intraperitoneally in order to test toxicity of mucin and broth alone without any fatalities being noted in either group.

Coagulase production was determined by tube test. Contents of an ampule of desiccated rabbit plasma (Difco) was suspended in 3 ml of distilled water and 2 drops of a 16 hour veal infusion broth culture of each strain under investigation was added to 0.5 ml of the plasma solution and incubated in a water bath at 37°C for 3 hours. A positive test was distinct clotting of the tube's contents. Presence of yellow to deep orange colonies after incubation at 37°C for 2 days and at room temperature near the light for 3 more days on a medium containing 2 parts 2% veal infusion agar and 1 part sterile homogenized milk indicated pigment production. Hemolysin production was manifest by distinct clear zones around bacterial colonies on 5% human blood agar plates within 72 hours at 37°C. Mannite fermentation was denoted by red colonies and the formation of a red color in the surrounding medium, after incubation at 37°C for 72 hours, on a veal infusion agar plate containing 1% mannite and Andrade's indicator.

Results. The significant enhancement in pathogenicity for mice achieved with the aid of gastric mucin with a considerable proportion of the bacterial strains studied is demonstrated in Table I. It may be noted from the table that whereas only 5 of the 60 strains were able to produce a mortality of at least 3 out of 5 mice without mucin, 42 proved lethal to the same degree with the adjuvant. Thus, by its use, the mouse was converted from a host resistant to staphylococcus infection via the intraperitoneal route into one readily susceptible with 37 strains. In no instance was any strain found pathogenic to mice without mucin and innocuous with it.

To facilitate comparison between *in vitro* and animal tests, all strains fatal to at least 3 out of 5 mice with mucin were arbitrarily classified as pathogenic and hence virulent. Accordingly, 42 of the 60 strains investigated were found virulent and 18 non-virulent. The number and proportion of microbial strains found mouse pathogenic or non-pathogenic in relation to their reactions to each of the presumptive *in vitro* tests is summarized in Table II. Examination of Table II reveals that although mouse pathogenicity with mucin was not in complete accord with the results of the biochemical tests, a degree of correlation ranging from high to fair did exist between both, depending upon the test. Excepting hemolysin production and varying with each criterion, strains reacting positively were generally pathogenic to mice, while the negative reactors were non-pathogenic in the main. As for hemolysin production, 73.8% of the 42 hemolytic strains proved pathogenic, as were 61.1% of the 18 non-hemolytic strains. The relationship between coagulase production and virulence as determined by the procedure reported upon is of particular interest for it provides experimental corroboration for the observation of Lack and Waillling(1), quoted above. Thus only 1 of the 11 strains that failed to produce coagulase proved pathogenic. On the other hand, though 41 of the 49 coagulase positive strains were pathogenic, the remaining 8 were found non-lethal to the experimental animals.

In light of the questionable validity of the biochemical indices in some instances and in view of a number of discrepancies noted between the *in vitro* reactions and mouse patho-

TABLE I. Enhancing Effect of Gastric Mucin upon Pathogenicity of 60 Strains of Staphylococci for Swiss Mice.

Mouse mortality*	Without mucin		With mucin	
	No. strains	% of total	No. strains	% of total
0/5	47	78.3	13	21.7
1/5	3	5.0	3	5.0
2/5	5	8.4	2	3.3
3/5	1	1.7	8	13.3
4/5	2	3.3	14	23.4
5/5	2	3.3	20	33.3

* Numerator = No. died. Denominator = Total No. animals inj.

TABLE II. Intraperitoneal Mouse Pathogenicity with Mucin of Staphylococci in Relation to Their *In Vitro* Reactions.

In vitro test	Reaction	Total strains	Mouse pathogenicity with mucin: Pathogenic No.	Pathogenic % total	Non-pathogenic No.	Non-pathogenic % total
Coagulase	Positive	49	41	83.7	8	16.3
	Negative	11	1	9.1	10	90.9
Pigmentation	Positive	39	34	87.2	5	12.8
	Negative	21	8	38.1	13	61.9
Hemolysin	Positive	42	31	73.8	11	26.2
	Negative	18	11	61.1	7	38.9
Mannite fermentation	Positive	52	41	78.9	11	21.1
	Negative	8	1	12.5	7	87.5

genicity with mucin, the animal virulence test reported upon is proposed as a supplementary method for directly assessing this property in staphylococci since it represents the determination of the capacity of a strain to induce death, which is the acme of virulence.

Summary. Significant enhancement of mouse pathogenicity was observed following intraperitoneal injection of staphylococci with gastric mucin. By means of the adjuvant, the mouse was converted from a host resistant to staphylococcus infection by this route into one readily susceptible with 37 strains. High to fair degree of correlation, depending upon the test, was found between the *in vitro* presumptive biochemical tests and intraperitoneal mouse pathogenicity with mucin but some discrepancies were noted between both. The procedure under consideration is proposed as a supplementary method for the direct determination of virulence of staphylococci.

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Comparison of Continuous and Strip Paper Electrophoresis Technics for Study of Serum Glycoproteins.* (22658)

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Paper strip electrophoretic technics are now widely used for fractionation studies of serum proteins. Application of the Hotchkiss-McManus technics for tissue polysaccharides to paper strips by Koiw and Gronwall(1) allows studies of carbohydrate components of the serum proteins. Technics for continuous paper electrophoresis apparatus using vertical curtains have been described by Grassmann and Hannig(2) and by Durrum(3). The use of such equipment allows the collection of serum fractions in quantities which make possible chemical analysis for the glycoprotein and protein components. The work described here was undertaken to compare results obtained by continuous electrophoresis technics with the less tedious paper strip methods.

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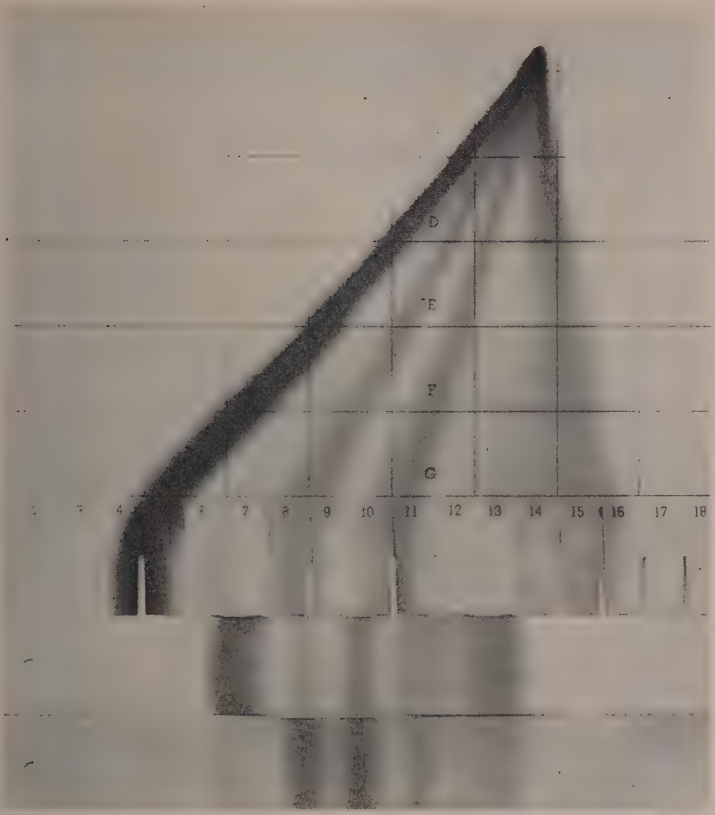

FIG. 1. Typical continuous and strip paper electrophoretic patterns. (Albumin is at the left in all patterns.) Top: Continuous electrophoretic curtain stained for proteins with bromophenol blue. Middle: Strip pattern stained with bromophenol blue. Bottom: Similar strip of same serum after development of periodic acid Schiff reaction for glycoproteins.

Methods. A Karler-Misco Continuous electrophoretic unit† was used with a constant voltage supply.‡ The plate supporting the paper curtain was found to make too much contact with the paper and was modified by making a plastic plate with fewer contact points. A slow stream of air was bubbled through the anode chamber to prevent plating out of buffer ions. A .045 barbital buffer (pH 8.6) was used for all studies. The apparatus was allowed to equilibrate for about 8 hours until the current became stable at 12 milliamperes. Electrophoresis was carried out at 350 volts for 24-48 hours. A paper wick (S and S No. 470) was used to feed the serum sample to the paper curtain.

At the end of each run the curtain was removed and the pattern developed with bromophenol blue in the same manner as used for strip electrophoresis. The collecting tubes, previously calibrated at 3 ml, were labeled and the volume in each adjusted to 3 ml. Aliquots were taken from each tube for estimations of protein by the biuret reaction and bound carbohydrate by the method of Shetlar, Foster, and Everett(4). Samples were also taken from each tube for paper strip electrophoresis. From these studies the protein constituents of each tube were identified. If more than one fraction was noted on the paper strip, it was scanned in the Spinco Analy-

† Microchemical Specialties Co., Berkeley, Calif.

‡ Model 1400 Research Specialties Co., Berkeley, Calif.

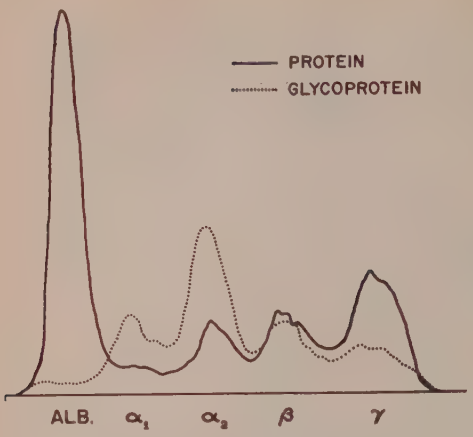

FIG. 2. Typical densitometer tracings of strip electrophoresis patterns for proteins and glycoproteins. Patient with active rheumatoid arthritis. Glycoprotein, 201 mg/100 ml; protein, 8.05 g/100 ml.

trol and the relative amounts of each fraction estimated.

Paper strip electrophoresis. Strip electrophoresis was carried out essentially by the method of Block, Durrum and Zweig(5) using Spinco hanging strip electrophoresis cells. 3MM filter paper was used for all work. Electrophoresis was conducted at constant current (5 milliamperes for 8 strips) using barbital buffer, .075M, pH 8.6. Duplicate strips, 10 λ for protein and 30 λ for glycoprotein studies, were made of each sample. For estimation of protein the strips were developed with bromophenol blue essentially as described by Block *et al.*(5). For glycoprotein studies, the strips were subjected to the periodic acid Schiff reaction as applied by Koiw and Gronwall(1) except that the reducing solution was prepared as described by Roboz *et al.*(6). The protein strips were quantitated with the Spinco Analytrol using blue filters; the glycoprotein strips were similarly evaluated with the same instrument using Klett 52 (green) filters. The total area under the protein curve was equated to the serum protein as determined by the biuret reaction, and the area under the glycoprotein curve was similarly made equal to the total serum glycoprotein obtained by the tryptophan method(4).

Results. Typical electrophoretic patterns by both technics are shown in Fig. 1, and typical analytrol curves for protein and glycoprotein are presented in Fig. 2. The difference in distribution of protein and bound carbohydrate is illustrated in both figures.

Average glycoprotein contents of serum fractions of patients with cancer and arthritis and for normal and pregnant subjects are summarized in Table I. The corresponding protein values are tabulated in Table II. Even with the small number studied, differences are apparent between the 4 groups.

In order to obtain some measure of the agreement between the two methods, the correlation coefficients for the 2 methods for the glycoprotein and protein of each fraction were calculated. Correlation coefficients for the glycoprotein moieties were: Albumin, .74; α_1, .81; α_2, .71; β, .78; and γ-globulin, .90; for the protein components they were: Albumin, .84; α_1, .75; α_2, .79; β, .55; and γ-globulin, .93. With the exception of β-globulin protein all of these correlation coefficients are significantly different from O at the 1% level of probability.

Discussion. Two different electrophoretic methods have been compared in this study. Not only do the electrophoretic technics vary, but the reactions used to quantitate the results are also different. For the continuous technic the protein of each fraction was estimated by the biuret reaction, while proteins by the strip method were estimated by densitometer readings made directly on the bromophenol blue dyed strip. Many studies have been made of the bromophenol blue staining technic as applied to paper strip electrophoresis(5) and a multiplicity of factors which affect the quantitation of protein fractions by this method have been noted. At present, it appears the best approach to use of this technic for study of serum fractions is an arbitrary one with standardized dyeing and scanning procedures. The continuous paper electrophoresis technic would appear to be more direct in its approach; however, this method is more difficult and is consequently unlikely to gain favor for extensive studies. The agreement of the continuous with the strip method

TABLE I. Distribution of Glycoprotein in Different Conditions.

Condition	No.		Bound carbohydrate* associated with						
			Albumin	α_1	α_2	β	γ	Total†	Sero-mucoid‡
Cancer	5	Strip	18	48	72	32	18	188	41
		Continuous	20	49	67	31	20		
Rheumatoid arthritis	2	Strip	15	38	80	35	30	198	27
		Continuous	18	30	79	35	38		
Late pregnancy	2	Strip	12	22	49	44	25	152	17
		Continuous	11	25	54	39	22		
Normal	3	Strip	12	18	36	33	24	122	15
		Continuous	13	16	35	31	24		

* mg/100 ml of serum.
† Bound hexose polysaccharide by tryptophan method(4).
‡ Determined by tryptophan method after isolation by method of Weimer *et al.*(7).

TABLE II. Comparison of Protein Distribution.

Condition	No.		Protein* associated with				
			Albumin	α_1	α_2	β	γ
Cancer	5	Strip	39.6	10.0	16.7	14.9	18.8
		Continuous	39.9	8.9	16.6	13.9	20.7
Rheumatoid arthritis	2	Strip	43.4	5.0	11.6	14.2	25.8
		Continuous	42.9	6.0	12.5	11.4	27.2
Late pregnancy	2	Strip	46.8	7.4	12.6	18.4	14.8
		Continuous	43.6	7.6	16.1	15.9	16.8
Normal	3	Strip	53.6	5.8	9.1	13.6	17.9
		Continuous	58.1	3.9	8.0	10.8	19.2

* Expressed as a % of total serum protein.

tends to strengthen the use of the latter method for routine work.

The agreement between glycoproteins by the two technics is rather surprising considering the great difference in technics and quantitation. The periodic acid Schiff reaction has not been evaluated as a quantitative test for serum glycoproteins, and the tryptophan method of Shetlar *et al.*(4) has not been thoroughly studied for purified serum fractions. However, the agreement noted in this paper tends to strengthen both of these technics as tools for investigations of glycoproteins.

Summary. A continuous paper electrophoretic technic was compared with a paper strip electrophoretic technic as a tool for the study of serum protein and glycoprotein fractions. Protein and glycoprotein for the continuous electrophoretic study were quantitated by analyses of the fractions by the biuret and tryptophan reactions. The glycoproteins by the strip technic were quantitated by the periodic acid Schiff reaction and the protein strips by the bromophenol blue staining. In both cases the strips were evaluated with an automatic photoelectric scanner. Good agreement between the two methods for both protein and glycoprotein was noted.

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Characteristics of the New Respiratory Viruses (Adenoviruses*) II. Stability to Temperature and pH Alterations.† (22659)

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To investigate the biologic characteristics of the respiratory viruses isolated independently by Rowe and co-workers(1) and by Hilleman and Werner(2) it was important to determine the stability of these agents under a variety of conditions. For practical considerations it was desirable to investigate the experimental conditions under which these viruses were inactivated, as well as the optimum and most practical conditions for handling and storing the agents. It is the purpose of this paper to present evidence that the 4 prototype viruses studied are relatively stable under varying conditions of pH and temperature.

Materials and methods. Tissue culture. Strain HeLa cells (Gey) derived from an epidermoid carcinoma of the uterus were employed(3). The methods used for the serial propagation of these cells and preparation of tube cultures were identical to those previously described(4,5). The nutrient fluid consisted of 40% human serum and 60% Hanks' balanced salt solution(3). *Viruses.* The viruses employed were types 1, 2, 3 and 4 of the new respiratory viruses(1,2) originally termed the Adenoid Degeneration or "AD" agents(1) or the RI-67 agents(2). More recently Huebner *et al.* have suggested the name adenoidal-pharyngeal-conjunctival (APC) viruses for this group of agents(6). A committee composed of interested investigators recently suggested the designation "Adenoviruses" for this group of agents(7), and this terminology will be employed hereafter. Types 1, 2, and 3 were kindly supplied by Dr. R. J. Huebner and type 4 (also termed RI-67(2) or ARD virus(4)) was furnished by Dr. M. R. Hilleman. The type designations are the same as those suggested by Huebner and co-workers (6). The viruses were propagated in HeLa cells using a maintenance mixture consisting of Scherer's MS 90%(3), and chicken serum 10% or MS 67.5%, chicken serum 7.5% and tryptose phosphate broth (Difco) 25%(5). Virus pools were prepared by freezing and thawing infected cells as previously described (5). Viruses were stored in sealed glass ampules at —70°C. *Infectivity titrations.* Serial 1:3.2 ($10^{-0.5}$) dilutions were prepared in Hanks' balanced salt solution, and 0.1 ml of each dilution was inoculated into each of 2 HeLa cell culture tubes. Inoculated tubes were incubated at 36°C for 6 days. A culture was considered infected when 50% or more of the HeLa cells had undergone specific cytopathogenic changes. The infectivity titer endpoint was calculated as the highest dilution of virus which infected one-half of the tubes inoculated. *Alterations of pH.* To adjust the viral suspensions to the desired pH, 0.5 N HCl or NaOH was employed. The quantity of HCl or NaOH required to obtain a desired pH was preliminarily determined using uninfected maintenance mixture. By this relatively crude method the number of drops of HCl or NaOH delivered by a calibrated capillary pipette in order to obtain a desired pH was placed in appropriate rubber stoppered tubes. To each tube was added 1.0 ml of virus infected maintenance mixture. After at least 5 minutes to permit complete mixture, the pH of each solution was determined electrophotometrically. The viral suspensions adjusted to the desired hydrogen ion concentrations were allowed to stand at room temperature for 30 minutes, after which the pH

* Name suggested to designate group of viruses previously termed AD, APC, or RI viruses(7).

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TABLE I. Storage of Adenoviruses at 4°C.

Virus	Virus infectivity titer after storage at 4°C (days) None	18	44	70
	(log)			
Type 1	−2.75	−2.75	−2.5	−2.75
2	−2.25	−2.5	−2.25	−2.25
3	−2.75	−3.0	−2.5	−2.75
4	−2.25	−1.75	−2.0	−2.0

TABLE II. Stability of Adenoviruses at Room Temperature (22-23°C).

Virus	Infectivity titer after storage at 22-23°C (days) None	2	4	7	10	14
	(log)					
Type 1	−3.5	−3.5	−3.5	−3.5	−3.5	*
2	−3.0	−3.5	−3.0	−3.0	−3.0	−2.5
3	−3.0	−3.0	−3.0	−3.0	−3.0	−3.5
4	−3.5	−3.5	−3.5	−3.5	−3.5	*

* Not tested.

of each was rapidly adjusted to pH 7.4-7.6 with 0.5 N HCl or NaOH. The total volume of NaOH and HCl which had been added to each tube had been recorded so that all viral samples, including the unaltered controls, were finally adjusted to the same volume with maintenance mixture. To determine viral inactivation, infectivity titrations were carried out on each aliquot in a given experiment at the same time. For one group of experiments a 0.1 M phosphate buffer at pH 6.5 was used to adjust the pH of the viral suspensions.

Results. Stability on storage. The 4 prototype viruses studied have been stored in the frozen state in glass sealed ampules at —70°C in a cabinet refrigerated with solid CO_2 and at —30°C in a mechanically refrigerated box for 19 and 7 months, respectively, the longest periods tested, without detectable decrease in infectivity titers.

It was next of considerable importance to determine whether the property of infectivity was stable at 4°C, ordinary refrigerator temperature. Pools of virus infected tissue culture homogenate were placed in screw-topped tubes at 4°C, and after 18, 44, and 70 days aliquots were removed and stored at —30°C until the completion of the test period. Infectivity titrations were done on all samples at the same time. The results of this experiment, summarized in Table I, indicate that these viruses can be stored at 4°C for over 2 months without loss of infectivity titer.

A similar experiment was carried out to determine whether the adenoviruses were inactivated when held at room temperature (from 22°-24°C) during the experimental period. The data from this experiment, presented in Table II, indicate that these viruses retained their infectivity titers at room temperature for 10 to 14 days.

Stability at 36°C. The above data indicated that the adenoviruses are relatively stable when stored over a wide range in temperature. The rate of inactivation of these agents when incubated at the temperature utilized for propagation of the viruses, *i.e.*, 36°C, was next determined. The results are summarized in Table III. For these experiments it was necessary to buffer the type 4 virus suspension in order to maintain a pH of 7.4. Preliminary experiments had shown that if the pH of type 4 virus preparations increased above 7.8 during incubation, the infectivity titer began to decrease within 24 to 48 hours; by 4 or 5 days the preparation would usually be non-infectious. As noted in Table III, however, when the pH of type 4 virus suspensions was maintained at a constant pH of 7.4 during incubation, this agent

TABLE III. Stability of Adenoviruses at 36°C.

Virus	Infectivity titer after incubation at 36°C (days) None	1	2	3	4	5	6	7
	(log)							
Type 1	−4.75	−4.75	−4.75	−4.0	−4.5	−4.5	−4.25 or >*	−4.25 or >*
2	−3.5	−3.75	−3.75	−3.5	−3.75	−3.25	−3.75	−3.5
3	−3.5	−3.25	−3.0	−3.25	−3.0	−3.0	−3.0	−3.0
4†	−3.25	−3.0	−3.0	−3.0	−3.0	−3.0	−2.5	−2.5

* Highest dilution tested, $10^{-4.0}$, infected both tubes.
† Buffered at pH 7.4 with 0.01 M phosphate buffer.

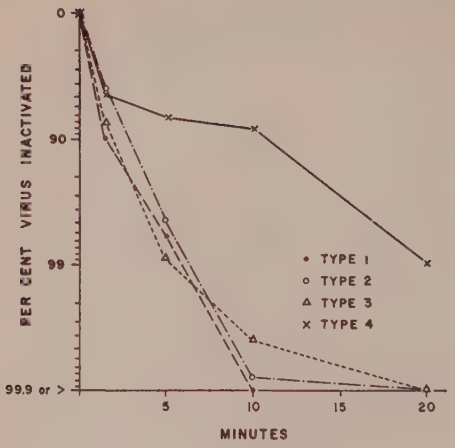

FIG. 1. Inactivation of adenoviruses at 50°C. Five 1.0 ml aliquots of each virus in tightly stoppered tubes were incubated in a 50°C water bath. At indicated intervals an aliquot of each virus was removed from the 50°C water bath and immediately immersed in an ice-water bath at 1°C. Infectivity titrations on all samples were done on the same day. Each endpoint represents geometric mean of 2 experiments.

was as stable as the other types. These data give further evidence of the marked stability of these agents, for when they were incubated at 36°C for as long as 7 days there was no measurable decrease of the infectivity titers by the methods employed.

Inactivation at 50°C and 56°C. To determine the rate and kinetics of the inactivation of the adenoviruses, the 4 agents under study were heated in 1.0 ml volumes at 50°C and 56°C for increasing periods. At the termination of the predetermined period of incubation the heated aliquot was immediately immersed in an ice-water bath at 1°C. Infectivity titrations on all samples of a single virus were done on the same day with a single lot of HeLa cell tubes. The results of 2 experiments with each virus are summarized in Fig. 1. Types 1, 2, and 3 viruses were inactivated at similar but not identical rates, whereas the type 4 virus was more resistant to inactivation at 50°C. It is of interest to note that the curves of inactivation of types 1 and 2 viruses could be interpreted to be straight line reactions. On the other hand, the curves of inactivation for types 3 and 4 viruses are not straight lines, which indicates that these agents were not inactivated at a uniform rate during successive unit time intervals at 50°C. No such differences in the rates of inactivation of these agents at 56°C were detectable, as all 4 viruses were more than 99.9% inactivated when incubated for only 2½ minutes and no residual infectivity could be detected after 5 minutes incubation at this temperature. That types 1-3 are noninfectious after incubation at 56°C for 30 minutes has been reported(6).

Degree of viral inactivation by varying pH. Multiplication of adenoviruses in HeLa cell cultures and the cytopathogenic alteration of the host cells induced by these agents is accompanied by an increase of lactic acid in the culture fluids(6,8,9) which may reduce the pH of the culture as low as pH 6.5 to 6.7(10). To determine whether the viruses may be inactivated during incubation of viral infected cultures the stability of adenoviruses was tested when the viral suspensions were adjusted to pH 6.5 and incubated at 36°C. These experiments clearly demonstrated that types 1-4 adenoviruses were not inactivated when incubated at 36°C for 24 hours at a pH of 6.5, conditions to which the viruses are often exposed during infection of HeLa cultures. To determine the stability of these agents under conditions which might be employed for chemical purification of viruses, as well as for other practical purposes, the inactivation of these agents over a wide pH range was also studied. The cumulative results of 2 or 3 experiments with each agent are summarized in Fig. 2. These data indicate that the adenoviruses are relatively stable at 22°-23°C between pH 6.0 and 10.0. It is of interest that type 4 virus was slightly less stable at acid pH's and type 3 virus was inactivated to a greater extent between pH 9 and 10 than were the other 3 prototype viruses studied. That these agents were not inactivated to a greater extent at pH's as low as 1.5-3.0 indicates that the infectious property is very stable under these unphysiologic conditions. Except for type 3 virus the agents under study were not inactivated to a significant degree between pH 7 and 10. At higher pH's, however, the viruses were readily inactivated,

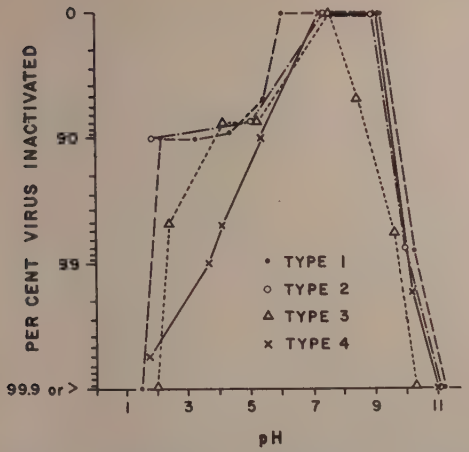

FIG. 2. Inactivation of adenoviruses by acid and alkaline pH changes. Adjustments of pH were made with 0.5 M NaOH or HCl. After pH change, all samples were held at 22-23°C for 30 min. pH of each aliquot was then adjusted to pH 7.4-7.6. Infectivity titrations on all aliquots in a single experiment were done on the same day. Each endpoint represents geometric mean of results from 2 or 3 experiments.

and at pH 11.0 infectious virus was not detected with any of the 4 types tested.

Discussion. The data presented indicate that the prototype viruses of types 1-4 of the new respiratory viruses(1,2), adenoviruses (7), are relatively resistant to inactivation both by heat and by pH changes. It must be emphasized, however, that only a single viral strain of each type was investigated; the variations of these characteristics within strains of a single type were not determined. Comparison of the types 1-4 viruses with the stability of other animal viruses indicates that their stability to heat and pH alterations is of the same order as the poliomyelitis(11-14) and Coxsackie viruses(15,16). All of these agents resist inactivation by ether(6,17-20). Further comparisons point out that the adenoviruses are considerably more stable to temperatures from 4°C to 50°C and pH alterations than are the influenza(21,22,23), mumps, and Newcastle disease viruses(24, 25). The stability of the adenoviruses when they are maintained at 4°C, room temperature (22°-23°C), or in the frozen state (—30°C to —70°C) facilitates attempts to isolate these agents from human sources. Furthermore, the capacity of these viruses to resist inactivation upon incubation at 36°-37°C eliminates a constant source of error from studies of the characteristics of the multiplication cycles(9,10) as well as other host-virus reactions(9,10).

The ecology of an infectious agent in many ways must be a reflection of the characteristics of the agent. That a virus can remain without inactivation of its important property of infectivity under a wide range of conditions should permit it to persist in nature either within or outside its host. These characteristics of stability similarly should permit the agents to spread from host to host under conditions which otherwise would make contagion difficult. Indeed, it may be considered that a basic understanding of the biologic and bio-physical characteristics of a virus not only facilitates insight into the activity of the agent in nature, but also permits hypotheses to be developed concerning the basic nature of the viral particle.

Summary. Types 1-4 of the new respiratory viruses, termed adenoviruses, were relatively stable when exposed to temperatures ranging from 4°C to 36°C. Type 1-3 viruses were inactivated in 10 to 20 minutes at 50°C, but the infectivity titer of type 4 virus was reduced less than 2 log units (99%) in 20 minutes. The adenoviruses were also resistant to inactivation at pH 6.0 to 9.0. The patterns of inactivation of the 4 viruses by alterations of pH as well as incubation at 50°C were similar, although consistent and significant differences among the 4 types were measured.

The excellent technical assistance of Miss Mary K. Dixon is gratefully acknowledged.

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Effect of Reduced Liver and Kidney Catalase Concentrations on Lethality of X Irradiation in Rats.* (22660)

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Hydrogen peroxide, which is toxic to mammals(1), is a product of the X irradiation of water(2). The high percentage of water in the body, coupled with the presence of the hydrogen peroxide-reducing enzyme, catalase, in most living organisms(3) suggests a possible interrelation of X irradiation, hydrogen peroxide, and catalase.

According to Feinstein *et al.*(4), about 75% of the total body catalase in Sprague-Dawley rats is present in the liver and another 7% in the kidneys. Heim *et al.*(5) have reported that a single injection of 1 g of 3-amino-1,2,4-triazole/kg of body weight in rats reduces the liver and kidney catalase concentration to about 11% of normal in several hours and that it takes more than 72 hr for the catalase to return to normal levels. It seemed of interest to determine whether reducing the liver and kidney catalase to low levels with this compound would increase the number of deaths in X-irradiated rats.

Materials and methods. Sprague-Dawley rats were used throughout. The 3-amino-1, 2,4-triazole (AT)† was used in concentrations of 50 mg/ml in water. Physiological saline (0.9%) was injected into all control animals. Both solutions were administered intraperitoneally in volumes of 20 ml/kg of body weight 4½ hr before either catalase assays or X irradiation. The results of the X-radiation experiments are shown in Table I. Inasmuch as the catalase-reducing effects of AT were reported for a different strain from that to those used in the experiments reported herein, the finding was checked. The liver catalase was assayed by the perborate method(6) and found to be depressed to 15-40% of that in

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† Generously supplied by the American Cyanamid Co.

TABLE I. Reduced Liver Catalase and Sensitivity to X Radiation (934 r).

Inj. material and amt inj. per kg rat wt	No. of rats inj. and wt range, g	No. of rats dying during 30-day observation period	Post-irradiation survival time, days
	Exp. I*		
AT, 1 g	12, 359–412	3	11 15 17
Saline (0.9%), 20 ml	12, 360–415	4	11 11 12 13
	Exp. II†		
AT, 1 g	20, 410–560	3	11 7 10
Saline (0.9%), 20 ml	20, 400–570	3	7 8 8

* Dose rate, 55.6 r/min.; target-rat distance, 89 cm.
† Dose rate, 51.4 r/min.; target-rat distance, 105 cm.

the controls.

The X-ray source was a General Electric Maxitron 250 operated at 250 kvp and 30 ma with a 3-mm Al filter and inherent filtration equivalent to 0.1 mm of Al (beryllium window). The hvl was 0.55 mm of Cu. A rotating circular lucite cage‡ (outside radius 17.5 cm) with 8 compartments was used as an exposure box. The cage was essentially the same in design as the circular mouse cage described by Upton *et al.*(7). Calibrations were made with a Victoreen 100-r condenser type ionization chamber, the chamber resting on a support in the cage and the thimble in the position of the center of the rat. The dose in both radiation experiments was 934 r; dose rates and vertical distances from the Victoreen to the level of the X-ray target were slightly different and are indicated in Table I.

Results. The catalase concentration was reduced to about 3% of normal 4½ hr after injection of AT, confirming a similar finding by Heim *et al.*(5) for Long-Evans rats. A check on the reduction of kidney catalase and the long recovery period for liver and kidney catalase also reported by these investigators does not seem necessary. The average value for liver catalase in the saline-injected control rats, 0.89 catalase unit, is very close to the 0.81 previously reported for the same strain (6).

Table I shows mortality data for the X-irradiated rats. In the first experiment, the radiation dose of 934 r resulted in a 30-day LD_{25} for the AT-injected animals and a 30-day LD_{33} for the saline controls. In the second experiment, the same level of radiation gave a 30-day LD_{15} for both groups. The data indicate that a low liver catalase level at the time of irradation does not increase the lethality of X radiation. Any possible role of the blood or blood-producing tissue catalase as a radiation protection agent is not elucidated by these experiments since AT does not reduce the blood catalase level(5) and since the catalase level in hematopoietic tissue was not measured. However, it seems clear that individual and strain differences in the lethal effects of X irradiation cannot be attributed to differences in liver and kidney catalase levels. It should be pointed out that the role of liver and kidney damage in 30-day irradiation mortality is not clear.

Summary. Liver and kidney catalase was markedly reduced in rats by the injection of 3-amino-1,2,4-triazole. X irradiation of these animals did not cause more deaths than in saline-injected control groups. It was concluded that differences in liver and kidney catalase levels cannot account for differences in sensitivity to the lethal effects of X irradiation.

‡ Kindly loaned by Dr. A. C. Upton.

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Specific Determination of Proline in Biological Materials.* (22661)

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Paper chromatographic studies on the amino acid metabolism of animal tissues cultivated *in vitro*(1) have shown that marked changes in the amino acid content of synthetic medium M 150(2,3) occur during the cultivation period. Medium M 150 contains 60 ingredients, including 21 amino acids, in a modified Tyrode's solution(4). Its complexity made it difficult to detect changes in certain amino acids, such as proline, that were present in low concentration or that reacted weakly with ninhydrin. Application of the isatin method for proline(5), or various modifications of this method(6-10), proved unsatisfactory since many other amino acids produced colors similar to that of proline. In earlier studies from this Laboratory(11-13), it was shown that the conventional ninhydrin procedure could be made specific for certain amino acids by treatment of the chromatograms with dilute acid or alkali subsequent to the ninhydrin development. Accordingly, similar procedures were tried with the isatin method and from these experiments, a specific and quantitative method for proline has been developed.

Methods. Samples of media for analysis (5.0 ml) were concentrated to dryness *in vacuo* over H_2SO_4 reconstituted in 0.2 ml of deionized water, and 10 λ portions used, without desalting. One-dimensional descending paper chromatograms were developed for 18 hours in either *n*-butanol-acetic acid-water or *n*-butanol-ethanol-water solvent systems. The chromatograms were then dried at 110°C for 2 to 3 minutes, dipped in isatin reagent (0.4% isatin dissolved in *n*-butanol containing 4% acetic acid), and reheated at 110°C for 10 to 15 minutes. Subsequent treatment of the chromatograms, with acid, to produce a selective color for proline is discussed in the succeeding paragraph. Full details of the general chromatographic procedures have been reported(1,11-13). Synthetic medium M 150 was prepared as described earlier(2,3). All chemicals and reagents were of the highest purity obtainable. Spectrophotometric measurements were made by cutting appropriate areas from the chromatograms and fixing them to the inner walls of 1 cm Corex cells filled with water. Optical densities were then determined in a Beckmann, Model DU. Direct measurements of the color intensity on the chromatograms were made with a densitometer (Welch Densichron).

Results. Selective development of color with proline and isatin. Paper chromatograms of medium M 150 were prepared, developed as described, and dipped in the isatin reagent. These chromatograms were then treated with various combinations of the acid and alkali procedures previously found suitable for specific color development with other amino acids(11-13). These experiments showed that washing the isatin-treated chromatograms with 1 N HCl, and suspending them at room temperature for 10 to 15 minutes intensified the blue proline chromogen, and at the same time caused almost complete fading of all other colored spots. Further

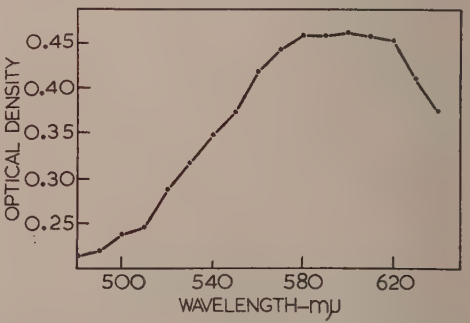

FIG. 1. Absorption curve of proline chromogen. Measurements made in Beckmann, Model DU, using 1 cm Corex cells containing area cut from developed chromatogram. 8 μg proline.

* The cooperation of Mr. C. E. Kerr, of this Department, in making the photographic preparations, is gratefully acknowledged.

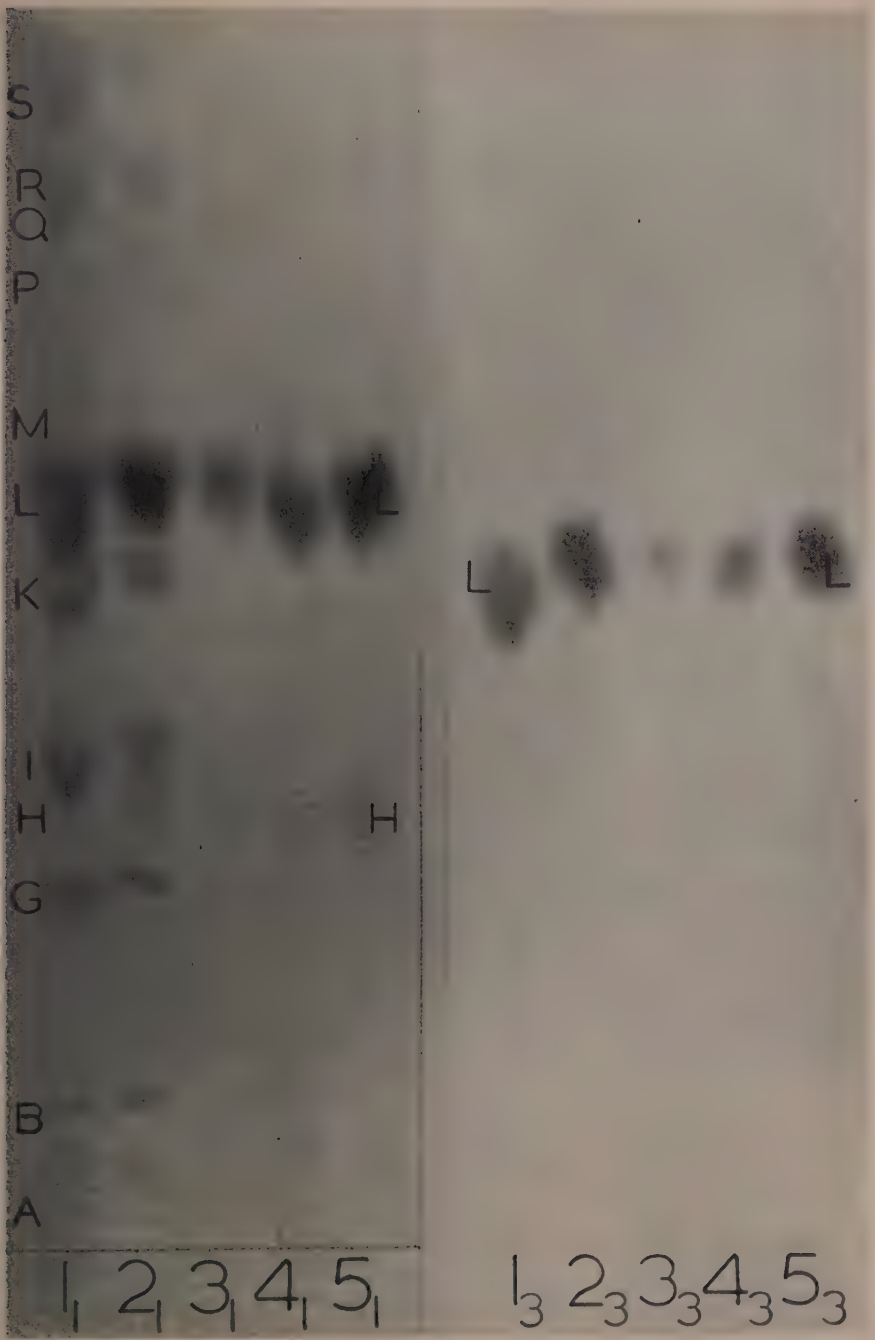

FIG. 2. Duplicate chromatograms, both developed by isatin, but chromatogram on right subsequently dipped into 1 N HCl and washed with water. 1_1 and 1_3, synthetic medium M 150, containing 20 μg of proline; 2_1 and 2_3, M 150 containing 25 μg of proline; 3_1 and 3_3, 5 μg of proline in water; 4_1 and 4_3, 10 μg of proline in water; 5_1 and 5_3, 25 μg of proline in water.

experiments established that this acid treatment bound the proline color so firmly to the paper chromatograms that the excess isatin reagent could be removed without eluting the proline chromogen. This was accomplished by washing with distilled water while the chromatograms were still damp. In this way, clear and distinct blue proline spots were obtained on a perfectly white background, free from any contamination with other amino acids. The absorption curve of this proline color is presented in Fig. 1 and shows a maximum at 600 mμ, with a broad plateau between 580 and 620 mμ. The absorption curve of the proline and isatin chromogen does not appear to have been reported previously.

Application of specific proline method to complex synthetic media. The application of this method to synthetic medium M 150 containing different levels of proline and hydroxyproline is illustrated in Fig. 2, showing 2 identical chromatograms, both developed by the conventional isatin procedure(5,6), but the second subsequently dipped into 1 N HCl and washed with water until the orange-yellow background color of the isatin reagent has been removed. By the conventional procedure (Fig. 2, 1_1 and 2_1), the presence of 20 and 25 μg of proline can be seen (L region), but interpretation of the chromatogram is made difficult by the large number of other amino acids also showing color development with isatin, and by overlapping of the proline spot with alanine (K) and tyrosine (M). Application of the new procedure (Fig. 2, 1_3 and 2_3) removes the interfering amino acids and proline is left as the only spot. The comparative behaviour of proline (L region) and hydroxyproline (H region) is also shown on these chromatograms. By the conventional isatin procedure (Fig. 2, 3_1 to 5_1), 5, 10, and 25 μg of proline show distinct color development, while the same quantities of hydroxyproline show only slight color formation. Application of the new procedure (Fig. 2, 3_3 to 5_3) clarifies the proline color development and, at the same time, completely removes the hydroxyproline color.

Quantitative measurement of proline by the new procedure. The gradation in color intensity with graded concentrations of proline (Fig. 2, 3_3 to 5_3) suggested that this procedure might be made quantitative. Attempts to elute the proline color with water, dilute HCl, dilute NaOH, methanol, ethanol, butanol, propanol, acetone, carbon tetrachloride, or benzene were unavailing, even though the extractions were performed with mechanical shaking until the filter paper was reduced to a pulp. Attempts were then made to determine proline directly on the chromatograms, employing a densitometer to measure peak density of graded levels of this amino acid. Following these measurements, the proline areas were cut from the chromatograms, placed on the inner walls of 1 cm absorption cells containing water, and the color intensity measured in a spectrophotometer at 600 mμ, employing as a blank a cuvette containing untreated filter paper. By careful placement of the proline spot to cover the center of the cuvette face, accurate measurements of peak density were obtained. The standard curves obtained by these two methods, based on the average results from 10 separate chromatograms containing graded amounts of proline, are presented in Fig. 3. By the spectrophotometer method (Fig. 3, Curve A), a linear response is exhibited over the concentration range of 1 to 16 μg. With the densitometer

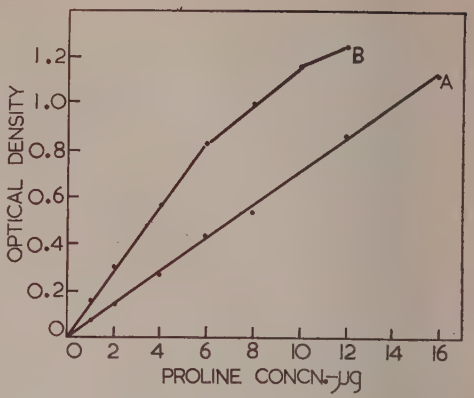

FIG. 3. Standard curves for graded concentrations of proline in M 150. Curve A, measurements made in Beckmann at 600 mμ. Curve B, measurements made on chromatograms with a densitometer. All points represent avg values from 10 separate chromatograms containing total amounts of proline specified.

method (Fig. 3, Curve B), linearity is maintained from 1 to 6 μg, then falls off. Attempts to maintain linearity by measuring both area and peak density were unsuccessful, and, for this reason, the spectrophotometer method is recommended for use.

Discussion. The isatin method has been used for determination of proline in known amino acid mixtures(5-10) but application of this method to complex biological media is difficult, since many other amino acids form colored complexes resembling that of proline. The present experiments have shown that the isatin method can be made specific for proline by subsequent treatment of the chromatograms with dilute acid. The chief advantages of this new procedure are simplicity and degree of specificity achieved. Moreover, use of this method with synthetic medium M 150 precludes interference by approximately 60 substances of biological importance, including amino acids, purines, pyrimidines, vitamins, and accessory growth factors. In addition, this method does not require desalting, even though the concentration of inorganic ions in the synthetic medium is high. Binding of the proline chromogen to the paper and fading of the isatin colors of the other amino acids resulting from the acid treatment makes it possible to apply this method under conditions of poor separation and resolution on the chromatograms. Quantitative studies have established that the intensity of the color is linear over a concentration range of 1 to 16 μg of proline. This range is sufficient to measure the levels of proline normally encountered in natural substances. The application of this method for proline, in conjunction with the specific method for hydroxyproline previously reported(13), makes it possible to determine selectively each of these amino acids in biological materials.

Summary. A specific method for determining proline is reported, based on treatment of isatin-developed paper chromatograms with dilute acid. None of the 20 other amino acids of synthetic medium M 150, including hydroxyproline, reacted by this procedure. The proline chromogen exhibits an absorption maximum at 600 mμ. The color is remarkably stable and can be used for the quantitative determination of proline over the range of 1 to 16 μg.

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Reaction of Enzymes of *Lactobacillus bifidus* var. *pennsylvanicus* with Bifidus Factor: Effect of Monosaccharides. (22662)

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Activity as an essential growth factor for *L. bifidus* var. *pennsylvanicus*(1) is exhibited by a number of substances of high or low molecular weight, but all containing N-acetyl-D-glucosamine. Among the low molecular weight compounds with comparably high microbiological activity are such different types as the alkyl-N-acetyl-β-D-glucosaminides and 4-O-β-galactopyranosyl - N - acetyl - D-glucosamine; active large molecules include the mucopolysaccharides of human milk and the blood group substances. Free N-acetyl-D-glucosamine itself has some growth-promoting effect but is of the order of 1 to 2% as active as the bound forms. A cell-free extract from *L. bifidus* var. *pennsylvanicus*(2) inactivates the various forms of bifidus factor with release of N-acetyl-D-glucosamine and the other constituent monosaccharides. Watkins and Morgan(3) found that the action of an enzyme preparation from *Trichomonas foetus* on blood group substances was inhibited by the presence of certain simple saccharides acting, presumably, in competition with corresponding structures in the blood group substances. These experiments suggested that further information about the essential structure and mode of action of the "bifidus factor" might be obtained by studying the effect of simple sugars, particularly those which are components of the bifidus factor, on the activity of the enzyme preparation from *L. bifidus* var. *pennsylvanicus*. In other experiments the direct effect of these sugars on the growth of *L. bifidus* var. *pennsylvanicus* was tested. A number of sugars, notably D-galactose, N-acetyl-D-glucosamine and L-fucose were found to inhibit the enzymatic decomposition of the mucopolysaccharides from human milk and hog gastric mucin. Most striking was the very marked effect of fucose with the bifidus factor of human milk. An inhibitory effect of fucose could also be observed *in vivo*. It markedly inhibited the growth of *L. bifidus* var. *pennsylvanicus* when the source of the bifidus factor was human milk but not when other forms of bifidus factor were used.

Materials and methods. Most of the enzymatic studies and *in vivo* microbiological tests were made with the bifidus factor of human milk and hog gastric mucin. In addition to skimmed milk itself, a deproteinized non-dialyzable fraction and a slowly-dialyzable fraction were tested. Hog gastric mucin was suspended in water and centrifuged to remove any insoluble portion. No purified fractions were used. For comparison with these high molecular weight forms of bifidus factor N-acetyl-D-glucosamine, 4-O-β-galactopyranosyl-N-acetyl - D-glucosamine and ethyl - N-acetyl-β-D-glucosaminide were used. The ethyl glycoside was present as approximately 20% of an α-β mixture. The α-form is inert in the test. The preparation of the cell-free extract of *L. bifidus* var. *pennsylvanicus* has been described(2). The lyophilized material contains about 20% of protein. In most of the experiments the concentration of the enzyme preparation was 2 mg per ml for milk or preparations from milk and 5 mg per ml for mucin. These concentrations gave approximately equal rates of inactivation of the two substrates. Human milk was tested in a 1 to 4 dilution, mucin and the non-dialyzable preparations from milk at 5 mg per ml and the dialyzable milk fraction at 7.5 mg per ml. Inhibitors were tested first at a level of 4%. In later experiments active substances were studied at higher and lower concentrations. All experiments were carried out at pH 6.0 in phosphate buffer. Samples were incubated at 37° under toluene. Aliquots were withdrawn at desired intervals, diluted to a concentration suitable for assay and heated 10 minutes in boiling water to inactivate the en-

TABLE I. Effect of L-Fucose and D-Galactose on Microbiological Inactivation of and Release of N-Acetylhexosamine from Bifidus Factor by Means of Bifidus Enzyme.

		Control		Fucose		Galactose	
Substrate	Hr	Activity,* % loss	N-acetyl-hexosamine,† %	Activity, % loss	N-acetyl-hexosamine, %	Activity, % loss	N-acetyl-hexosamine, %
Non-dialyzable fraction from human milk	1	10	30	3	19	2	10
	6	72	68	20	44	27	42
	19	89	90	53	54	64	70
	48	93	93	60	75	78	90
Hog gastric mucin	1	28	24	10	10	4	8
	6	31	50	16	34	4	29
	19	48	72	31	51	25	34
	48	89	89	73	76	64	64

* Both substrates had an initial microbiological activity of approximately 2 units/mg.
† N-acetylhexosamine values are calculated as % of total amount which is released when substrate is completely inactivated.

zymes. N-acetylhexosamine was determined by a modification of the method of Aminoff *et al.*(4). The procedure for the microbiological assay of bifidus factor has been reported(5). The basal medium containing enzymatically digested casein corresponded to that of Hassinen *et al.*(6) modified by the addition of 1 g of "Tween 80" per liter of double strength medium. The effect of sugars, particularly fucose, was tested not only with *L. bifidus* var. *pennsylvanicus,* but also with the regular strain which does not require bifidus factor(7).

Results. Galactose and fucose inhibited the enzymatic splitting of the polysaccharides of both human milk and gastric mucin. A typical experiment is shown in Table I. Both release of N-acetylhexosamine and loss of microbiological activity were delayed. In other experiments the effect was shown to be proportional to the amount of galactose or fucose present. Similar results were obtained with lactose which is hydrolyzed by the bifidus enzyme preparation. The effect of N-acetyl-D-glucosamine could not be as conveniently studied. The amounts of sugar used as inhibitor were much larger than those which would be released from the substrates so that N-acetyl-hexosamine could not be measured. Also, since N-acetyl-D-glucosamine is a growth stimulant it was necessary to remove it from the reaction mixture by dialysis before estimation of microbiological activity. The results of the experiments carried out indicated that N-acetyl-D-glucosamine did act as an inhibitor of the bifidus enzyme.

Galactose and N-acetyl-D-glucosamine had a greater inhibiting action with mucin than with human milk fractions. In many experiments in which the release of N-acetylhexosamine was followed the galactose effect with human milk was limited chiefly to the first hours of the experiment whereas with mucin it was quantitatively greater and more prolonged. With N-acetyl-D-glucosamine the rate of inactivation of milk polysaccharides was only moderately slower than that of the controls. With mucin the effect of N-acetyl-D-glucosamine seemed more persistent. Even after prolonged incubation about 30% of the initial activity was left while the control was completely inactive (Table II). Fucose, however, was most active with the milk polysaccharides and the extent of inhibition was greater than any observed effect of galactose or N-acetyl-D-glucosamine. This observation was confirmed in repeated tests. The effect appeared to depend primarily on the concentration of fucose rather than on a competitive action with the substrate. Marked inhibition of release of N-acetylhexosamine had been shown with 4% fucose and 0.5% of the non-dialyzable fraction of milk. With lower levels of fucose, 0.06 to 2%, inhibition was demonstrated with levels of substrate from 0.15 to 5%.

Of a number of other sugars tested only methyl-α-L-fucopyranoside and l-acetamino-

TABLE II. Effect of N-acetyl-D-glucosamine on Inactivation of Bifidus Factor by Bifidus Enzyme.

Substrate	Hr	Activity (%) Control	N-ac-glucosamine
Non-dialyzable fraction from human milk	1	79	88
	6	32	41
	24	17	30
	48	<5	11
	80	<5	<5
Hog gastric mucin	1	82	89
	6	62	62
	24	49	57
	48	<5	56
	80	<5	31

lactose were active. D-Glucose, L-fructose, L-rhamnose and L-arabinose were essentially without effect.

In the microbiological assay of samples from the enzyme tests it became apparent that, in some cases, the presence of L-fucose was inhibiting the growth of *L. bifidus* var. *pennsylvanicus*. When this observation was tested systematically, with varying amounts of fucose in the medium and with different forms of bifidus factor, it was found that inhibition occurred when bifidus factor was supplied as human milk or fractions from human milk, but not when other forms of bifidus factor were used (Table III). The effect was appreciable with 0.1% of fucose in the medium and increased with larger amounts of fucose. The effect was greater with suboptimal levels of bifidus factor. Fucose was equally effective whether it was autoclaved with the medium or added aseptically to previously autoclaved medium. Methyl-α-L-fucopyranoside which inhibited the bifidus enzyme had no effect *in vivo*. Up to a level of 0.5% fucose did not inhibit or retard the growth of the regular, non-milk-requiring strain of *L. bifidus*. The medium for *L. bifidus* contains lactose as carbohydrate. The organism does not grow as well with either glucose or galactose. These monosaccharides were tested for possible inhibitory effect. No inhibition was found when they were added in concentration up to 1%.

Discussion. The mucopolysaccharide of human milk, like the blood group substances, contains N-acetyl-D-hexosamine, D-galactose and L-fucose. It differs from the blood group substances in that it contains glucose and that the hexosamine is almost entirely glucosamine while all of the blood group substances have an appreciable proportion of galactosamine. Structure of a hexasaccharide unit of the milk polysaccharide has been reported by Kuhn(8) but the position of this unit in the substance as it occurs in milk is still to be worked out. The hexasaccharide contains 2 molecules of L-fucose in α-fucopyranosidic linkage. The L-fucose of the blood group substances is also present as an α-fucopyranoside, but its position in the whole oligosaccharide is not known and is probably not the same for all of the blood group substances (*cf* 9). Kuhn and Osman(10) have shown that the fucose-containing oligosaccharides of human milk have very low blood group O(H) activity not only in comparison with the blood group substance but also in comparison with simple α-fucopyranosides and L-fucopyranose.

In earlier experiments with the *L. bifidus* enzyme preparation loss of microbiological activity was known to be accompanied by increase of reducing sugar, and release of N-acetyl-D-glucosamine was revealed chromatographically. In the present experiments release of N-acetyl-D-hexosamine in a form measurable by the Morgan-Elson reagent, was followed quantitatively and was found to parallel closely the degree of microbiological inactivation with enzyme alone or in the presence of galactose or fucose. Approximately 75 γ of N-acetyl-D-glucosamine was released for each unit of activity lost. Of the 4 components of the mucopolysaccharides tested only glucose was without effect. The other sugars exhibited the type of inhibition which might be expected from the presence of an

TABLE III. Growth of *L. bifidus* var. *pennsylvanicus* with and without Fucose.

Supplement of bifidus factor	Acid production (ml of N/10) No fucose	0.15% fucose
Skimmed human milk, .06 ml	8.5	5.3
Non-dialyzable fraction from human milk, .5 mg	10.5	8.7
Dialyzable fraction from human milk, 1.5 mg	7.5	5.0
Hog gastric mucin, .5 mg	9.7	9.4

end product. Only in the case of fucose with the milk polysaccharide was there indication of a more specific effect. *In vivo* only fucose exerted an inhibitory effect. This inhibition was limited to the milk polysaccharide and could not be demonstrated with hog gastric mucin or low molecular weight forms of bifidus factor. Neither did fucose have any effect on the growth of strains of *L. bifidus* which do not require the bifidus factor. The position of fucose in the milk polysaccharide appears to give it distinctive properties with respect to activity as bifidus factor.

Summary. 1. The effect of the constituent monosaccharides on the enzymatic inactivation of high molecular forms of bifidus factor has been studied. The most marked effect was observed with fucose when it was used with human milk or purified fractions from human milk. 2. The significance of this effect of fucose on the inactivation of the bifidus factor *in vitro* was emphasized by the experiments *in vivo*. L-Fucose markedly inhibited growth of *L. bifidus* var. *pennsylvanicus* when the source of bifidus factor was human milk, but not when it was supplied as mucin or the simple N-acetyl-D-glucosamine-containing compounds.

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Chemical Inhibitors of Theiler's Virus.* (22663)

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Certain amino acids, nucleoprotein derivatives and analogues as well as various other compounds, previously have been reported to inhibit propagation of Theiler's GD VII virus, *in vitro*(1-5). In the present study some additional chemicals have been tested. A preliminary report was given elsewhere(6).

Methods. Tissue cultures of minced brain tissue from newborn mice were made in 50 ml stoppered, Erlenmeyer flasks. Each flask contained 50-100 mg of tissue in 3 ml Simms' solution at pH 8-9 and approximately 100 intracerebral MLD_{50} of a tissue culture passage strain of Theiler's GD VII mouse encephalomyelitis virus. After incubation at 35-36°C for 2 days the pooled supernatant fluids obtained by centrifugation of the contents of 3 flasks were tested for viral content by hemagglutination of human red cells. Ordinarily titers of 1280-2560 are obtained by this procedure. The structure of the hydroxycytidine used as prepared in this laboratory has not been established with certainty. The Metuchen was kindly furnished by Dr. F. M. Berger, Wallace Laboratories, the thiosemicarbazone compounds by Dr. R. L. Thompson, Sterling-Winthrop Research Institute.

Results. Table I gives results of tests with various chemicals. Of the substituted pyrimidine nucleosides, the cytidine compounds are slightly more inhibitory than are the corresponding derivatives of uridine. Some of the naturally occurring deoxyribosyl nucleo-

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TABLE I. Percentage Reduction of Hemagglutination Titers of Virus in Presence of Certain Compounds.

Compound	% reduction of titer	
Nucleosides	.5 mg/ml	.1 mg/ml
5-bromocytidine	98	0
5-chlorocytidine	95	0
5-hydroxycytidine	90	50
5-hydroxyuridine	75	0
deoxyadenosine	75	0
deoxycytidine	0	—
deoxyguanosine	75	50
	3 mg/ml	1 mg/ml
5-bromodeoxyuridine	—	0
5-hydroxydeoxyuridine	—	0
thymidine	0-50	0
deoxyadenosine	100	95
deoxycytidine	50	0
deoxyuridine	—	0
Pyrimidine derivatives		
deoxycytidine-5′-phosphate	—	0
2-amino-4 methylpyrimidine	100	0
4,6-diamino-2-thiopyrimidine	90	85
2,4-dichloropyrimidine	0	—
2,4-dichloro-6-methylpyrimidine	0	—
5-bromouracil	—	50
2-thiouracil	50	0
propylthiouracil	100	75
5-methyl-2-thiouracil	90	0
6-methyl-2-thiouracil	100	0
Thiosemicarbazones	.1 mg/ml	.01 mg/ml
butane-2,3-dione-2-methoxime 3-	85	50
2-thenaldehyde-	50	0
5-bromo-2-thenaldehyde-	—	0
2,3-butane oxime-	—	0

Other chemicals	.1 mg/ml	.05 mg/ml	.005 mg/ml
chlorpromazine	—	100	50
quinine dihydrochloride	100	—	0
bacitracin	90	50	—
polymyxin, penicillin, erythromycin	50	—	—
mycostatin	0-50	—	—

sides were inhibitory as were also some of the ribosyl nucleosides(3). The compounds listed under pyrimidine derivatives varied in their capacity to inhibit virus. Two of the 4 thiosemicarbazones tested were inhibitory. The more active of the 2 was previously found by others to be the most inhibitory for vaccinia virus(7). Of the other chemicals used, chlorpromazine and one not listed in Table I, Metuchen (2,3 - diethyl - 3 - propanediol dicarbamate), are known to have effects on the CNS. The latter compound was not active at a concentration of 0.1 mg/ml but did give 75% reduction in hemagglutination titer at a concentration of 1 mg/ml and complete inhibition at 3 mg/ml. Chlorpromazine was as inhibitory as any drug ever tested in our system. An *in vivo* trial of the drug was made in the following manner. Groups of 10 albino Swiss mice (10-15 g) were each given intraperitoneally (i.p.) 1 mg of chlorpromazine daily (2 mg daily is lethal). A control group of mice received saline instead of chlorpromazine. Seven hours after the first dose of drug and immediately prior to the second dose, each mouse was given an intracerebral (i.c.) injection of 0.03 ml of tissue culture virus (approx. 1-10 MLD_{50}). The test mice died 3 to 8 days after virus injection as follows 3,3,3,3,7,7,7,7,7,8; deaths of control mice were at 4,7,7,7,8,9,10 days and 2 survived. In a second experiment drug treatment was given as above daily beginning on the third day after virus inoculation. Mice died on the days indicated after virus injections; test mice, 5,5,6,7,7,10,11, 3 survivors; control mice, 4,6,7,7,10,10,10, 2 survivors. The drug did not protect mice from viral death. The early deaths in the first experiment possibly resulted from a combination of viral effect and drug toxicity.

Bacitracin polymyxin, penicillin and erythromycin were found to inhibit virus; streptomycin, tetracycline, oxytetracycline and chloramphenicol did not inhibit. Mycostatin irregularly gave inhibition; possibly this irregularity is associated with the poor solubility of the drug. Polymyxin was used to treat mice which had received 1 MLD_{50} of virus i.c. 3 days previously. The mice were given 0.2 mg i.p. daily; control mice received saline. Control and test mice died 5-9 days after virus injection. The drug did not affect the infection in mice. Under the conditions of this experiment 0.3 mg daily of polymyxin was a fatal dose.

Inhibition of virus by 5-hydroxyuridine was reversed by uridine-5′-phosphate as shown in Table II. Comparable reversal of this inhibition also occurred with uridine(3).

Glutamine has been found by others(8) to be necessary for the propagation of poliovirus

in HeLa cell tissue cultures. This compound inhibited GD VII virus in our tissue culture system. The hemagglutination titer was reduced 75% by a concentration of 0.1 mg/ml.

Discussion. Uridine does not inhibit virus and partially reverses inhibition produced by some substituted uridine nucleosides(3). As shown above uridine-5′-phosphate also reverses the inhibitory effect of the nucleoside derivative, 5-hydroxyuridine. The greater activity of cytidine derivatives in comparison with uridine derivatives as shown here is similar to the findings for *Neurospora*(9). Although chlorpromazine was active, *in vitro*, it failed to influence the infection in mice. The quantity used in the tests was nearly the maximal amount tolerated since it was found in preliminary studies that 2 mg daily was a lethal dose. The dose of 1 mg used resulted in generalized muscular weakness from which recovery was gradual and not complete as long as 24 hours later. The drugs tested probably did not act by interfering directly with the hemagglutination test since control tests with representative drugs from each group and virus incubated for 1 hour at room temperature gave the same hemagglutination titer as virus alone with one partial exception; the RBC sediment pattern in the presence of 0.1 mg/ml quinine dihydrochloride did not have as definite an endpoint as the control tube with virus.

Summary. Certain pyrimidine-related-compounds inhibit Theiler's virus, *in vitro*. Inhibition by 5-hydroxyuridine is partially reversed by uridine-5′-phosphate. Of various other chemicals found to inhibit virus, *in vitro*, chlorpromazine and polymyxin did not protect mice from viral infection.

TABLE II. Reversal of 5-Hydroxyuridine Inhibition by Uridine-5′-phosphate.

	% reduction of hemagglutination titer
5-hydroxyuridine, .5 mg/ml	75
Uridine-5′-phosphate, .5 mg/ml	0
5-hydroxyuridine, .5 mg/ml + uridine-5′-phosphate, .5 mg/ml	50
5-hydroxyuridine, .5 mg/ml + uridine-5′-phosphate, 1 mg/ml	0

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Relation of Endocrines and Clearing Factor Inhibitors to Hyperlipemia in Fasted Animals.*† (22664)

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A variety of substances inhibit the clearing action of lipoprotein lipase *in vitro*. These include protamine(1), polylysine(2), diisopropylfluorophosphate (DFP)(3), iodinated thyronines(4), Na cholate(5), and an inhibitor (LM) present in plasma of rats receiving cortisone(6). Of these protamine(7), polylysine (2), Na cholate(5), and LM(8) have been reported to produce hyperlipemia when injected

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into intact animals, presumably by inactivating lipoprotein lipase *in vivo*. LM, apparently produced by the pituitary, induced hyperlipemia in adrenalectomized and hypophysectomized rats and may be considered to either activate the fat depots or to inhibit lipoprotein lipase. It was of interest therefore to establish whether other hyperlipemia inducing agents act similarly. The failure of these to produce hyperlipemia in the absence of the adrenals or pituitary would suggest that the effect in intact animals could not be due to direct inactivation of lipoprotein lipase *in vivo* but rather the result of mediation through release of LM.

Materials and methods. To demonstrate the effect of food intake on the hyperlipemia inducing action of LM, 6 groups of 5 male and 5 female Wistar strain rats weighing 125-150 g were used. Three groups were maintained on the laboratory basal diet of Purina Chow checkers containing 5% fat up to the time of injection (Category A) and 3 groups were fasted for 20 hours preceding injection (Category B). Neither category received food from the time of injection until bled. One group of each received 5 mg LM/kg either IV, IP or IM. Blood samples were obtained at the end of 2 hours. For the other rat studies all animals were fasted 18-24 hours and all injections were made into the recurrent saphenous vein. Solutions of compounds to be tested were freshly prepared in physiological salt solution just prior to use and the concentration was adjusted so that 0.1 ml/100 g of body weight was administered. Blood was obtained by cardiac puncture through the open chest while the animal was under light ether anesthesia. At this time it was verified that the stomach of fasted rats contained no food. The blood was mixed with 0.1 M Na citrate (1:9) and centrifuged in a constant temperature room (5° ± 1°C) until separation of the plasma occurred. Total cholesterol was determined by the method of Schoenheimer-Sperry(9), total fatty acids by that of Stoddard and Drury(10), and lipid P by that of Fiske and SubbaRow(11). Adrenalectomized rats maintained on Purina Chow checkers and 0.9% NaCl in their drinking water, and hypophysectomized rats fed white bread, Pard dog food, and oranges were used 10-14 days after operation. Completeness of adrenalectomy and hypophysectomy was checked in each rat at autopsy. In another study 3 male and 3 female mongrel dogs previously fasted for 18 hours were injected intravenously as described below. The previous diet of these dogs contained approximately 5% fat. Lipid values were determined, as described for the rats, on blood samples obtained from the recurrent saphenous vein before, during, and after each experiment. At least 7 days were allowed between studies and blood samples were drawn during these intervals.

Protamine sulfate (Lilly) of fish origin was administered to 20 fasted intact rats, 20 fasted adrenalectomized rats, 20 fasted hypophysectomized rats and to the dogs in doses of 10 mg/kg. Blood samples were obtained from rats 1 hour after the injection and from dogs at hourly intervals for 6 hours. DFP supplied by Drs. John W. Gofman and Bernard Shore was administered to dogs in doses of 0.05, 0.10, and 1 mg/kg and to 7 fasted intact rats, 7 fasted adrenalectomized rats and 7 fasted hypophysectomized rats in a dose of 1 mg/kg. Blood samples were obtained from the dogs at hourly intervals for the first 6 hours after injection and at the 24th hour and from rats 2 hours after injection. Pseudomonas polysaccharide (Piromen, Travenol Laboratories) was administered to 20 fasted intact rats, 20 fasted adrenalectomized rats and 20 fasted hypophysectomized rats in a dose of 50 μg/kg. Blood samples were taken 2 hours after the injection. The lipid mobilizer (LM) was prepared from the plasma of cortisonized horses as previously described(8) and was administered to 20 fasted intact rats, 20 fasted adrenalectomized rats and 20 fasted hypophysectomized rats and to the dogs in 1 mg/kg doses. Blood samples from rats receiving LM were obtained 1 hour after injection and from dogs at hourly intervals for the first 6 hours and at the 24th hour.

Results. Table I shows that injections of LM produced hypercholesterolemia only

TABLE I. Effect of Food Intake on Hypercholesterolemia Produced by LM in Rats.

	Total plasma cholesterol (mg %)*		
	IV	IM	IP
Fasted	184.3 ± 3.5	148.6 ± 3.2	168.6 ± 3.6
Non-fasted	60.0 ± 2.6	61.8 ± 2.0	64.0 ± 1.8

* Avg 10 rats/group ± S.D.

when injected into fasted rats but not in those which had received food up to the time of injection. The hyperlipemia in fasted rats receiving LM is illustrated in Fig. 1 and the values for each lipid component represent the averages for 20 rats (S.D. ± 5%). The hyperlipemic action did not depend upon the presence of either adrenals or pituitary. Fig. 2 shows the degree of hyperlipemia in rats receiving protamine. Similar results were obtained with rats administered either DFP or piromen. As with LM, in intact rats they produced a 2-3 fold elevation of all classes of lipids and a marked increase in optical density. In contrast, however, neither protamine, DFP nor piromen produced hyperlipemia in the absence of either adrenals or pituitary. The rats administered the DFP had convulsions and those given piromen had elevated rectal temperatures of at least 2.5-3°C. In the dogs receiving LM there was a 2-3 fold increase in all lipid components in 90-180 minutes which persisted for at least 3 hours. The plasma lipid values were in the normal range 24 hours after injection. Protamine administered to these same dogs at a later date also produced an increase in plasma lipid values, but the effect was less intense, of shorter duration, and the dose required was 10 times greater than that of LM. Similar administration of DFP to these dogs also produced hyperlipemia resembling that produced by protamine, but this occurred only after convulsant doses. The hyperlipemia did not appear after the dogs were administered non-convulsant doses of DFP although they exhibited unequivocal signs of anticholinesterase effects.

Discussion. LM induced hyperlipemia only in animals that were primed for lipid mobilization by starvation. It should be pointed out that all animals used in our studies were maintained on a low fat diet. In previous investigations of lipid mobilization diet, degree of fasting, or pretreatment with glucose had marked influences on the response (12). Of the many substances that produce hyperlipemia, particular significance has been attached to protamine(7) because this compound is considered to be a specific antagonist of heparin. Therefore the hyperlipemia it induces has been attributed to inactivation of lipoprotein lipase and has been presented as

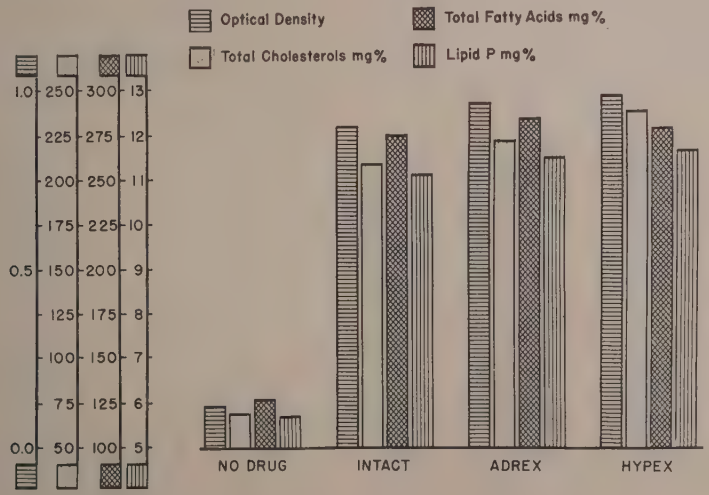

FIG. 1. Hyperlipemia by 1 mg LM/kg IV in rats (1 hr $\bar{p}$ inj.).

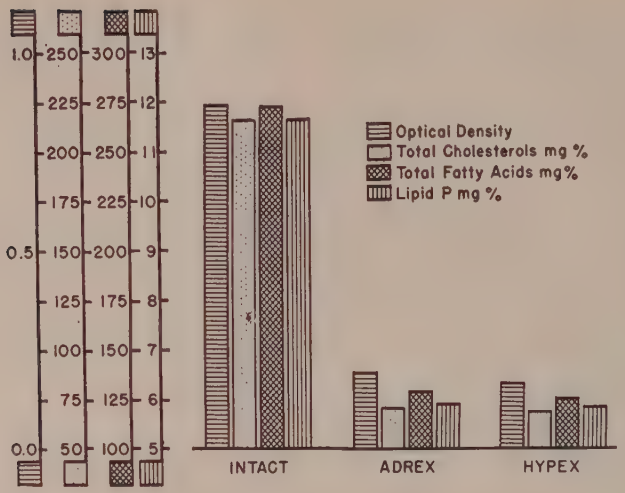

FIG. 2. Hyperlipemia by 10 mg protamine SO_4/kg IV in rats (1 hr $\overline{p}$ inj.).

evidence for the presence of heparin in the enzyme. However, the effective dose of protamine(7) is far in excess of that needed to neutralize the amounts of circulating heparin that can be detected by present methods and is within the toxic range that activates the pituitary and adrenals(13). Of particular significance is the large dose of protamine required to produce the hyperlipemia which was less intense than that produced by LM. Administration of larger doses resulted in more intense hyperlipemia which was accompanied by gross hematuria, prolonged shock with terminal asphyxial convulsions. The failure of protamine to produce hyperlipemia in adrenalectomized and hypophysectomized rats establishes that the action was not due to a direct effect on lipoprotein lipase but was mediated through the adrenal-pituitary system. Spitzer has also presented indirect evidence that protamine hyperlipemia involves more than anti-heparin activity(14). Shore has demonstrated that DFP is a potent inhibitor of lipoprotein lipase *in vitro*(3). Therefore administration of DFP in amounts to achieve similar concentrations in the plasma should result in hyperlipemia as a consequence of inactivation *in vivo*. We could observe no hyperlipemia in intact animals until convulsions had occurred which required administration of several times this amount. Furthermore, the hyperlipemia did not occur in adrenalectomized or hypophysectomized rats receiving the same doses and exhibiting convulsions. These results establish that the hyperlipemia due to DFP is a non-specific one mediated through the adrenals and pituitary and is not related to its anti-lipoprotein lipase activity. Since LM was obtained only from animals with intact pituitaries and induced hyperlipemia in the absence of either the adrenals or pituitary, it may be assumed to be released through the pituitary and to be the mediator through which stressors act as hyperlipemic agents. Administration of heparin releases lipoprotein lipase, but more direct evidence than hyperlipemia produced by protamine or DFP is needed to establish the presence of a heparin moiety in this enzyme or that it is involved in the physiologic or pathologic transport of lipids. The results with piromen establish that this agent also induced hyperlipemia by activating the pituitary and adrenals. Piromen has not been shown to inactivate lipoprotein lipase and therefore furnishes an excellent example of a non-specific stressor activating the pituitary-adrenal system to produce hyperlipemia.

Summary. 1. LM produced hyperlipemia only in animals primed for lipid mobilization. 2. Intravenous injection of toxic doses of pro-

tamine sulfate, convulsant doses of diisopropylfluorophosphate and pyrogenic doses of piromen produced hyperlipemia in intact animals. 3. The same doses of these substances injected into adrenalectomized and hypophysectomized rats did not produce hyperlipemia. 4. The hyperlipemic action of protamine or DFP is not attributable to direct inhibition of lipoprotein lipase but to a secondary effect mediated through the adrenals and pituitary. 5. The hyperlipemic action of protamine, DFP, pyrogens and possibly other stressors is best explained by release of LM.

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Evaluation of Germicidal Efficiencies of a Group of Antibiotics Tested by Tissue Culture Technic. (22665)

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An improved one-step tissue toxicity technic was proposed recently(1) for evaluation of germicides intended for clinical application.

The germicides were tested for their effect on living embryonic chick heart tissue fragments as well as for their ability to kill bacteria. A number known as the Toxicity Index was calculated from the results, which was defined as the ratio of highest dilution of germicide required to prevent growth of the tissue fragments in 10 minutes to highest dilution required to kill the test bacteria in the same period and under similar conditions. Theoretically an index less than one means that the germicide is more toxic to the test organism than to the embryonic tissue fragments; an index greater than one means that the germicide is more toxic to the tissue fragments than to the bacteria. The smaller the index the more nearly perfect the chemotherapeutic agent. There is no relation whatsoever between a phenol coefficient and a toxicity index.

The same procedure was followed for evaluation of the germicidal efficiencies of a group of antibiotics including: Bacitracin, Chloramphenicol (Chloromycetin) Penicillin G sodium, Polymyxin B sulphate, Streptomycin sulfate, and Terramycin HCl.

Aureomycin (Chlortetracycline HCl) and Achromycin (Tetracycline HCl) precipitated from saline shortly after being prepared and could not be evaluated. Likewise, Erythromycin failed to dissolve in saline.

Methods and materials. Embryonic chick heart tissue fragments from embryos 12 days old were used. Hearts were selected because of the ease by which a constant source of tissue could be obtained. The hearts were removed from the embryos and minced into fragments 0.5 to 1.0 mm in diameter by means of a sharp knife. The fragments were

washed twice in normal saline, then resuspended in saline in the proportion of one heart per ml. This suspension of tissue fragments was handled like a broth culture of bacteria.

Micrococcus pyogenes var. *aureus* is generally used as the organism in germicide testing but since it liquefied plasma it could not be used here; *M. pyogenes* var. *albus* was used instead. Unless the fragments are embedded in coagulated plasma, microscopic observations of Carrel flasks for tissue proliferation could not be made.

The culture was grown on FDA nutrient agar slants incubated at 37°C and transferred daily. The organisms were washed from a 24-hour culture with saline and the suspension standardized in a photometer to correspond to the same turbidity as a No. 1 McFarland nephelometer standard.

For each test, a series of 8 dilutions of antibiotic was prepared in screw cap test tubes measuring 20 x 150 mm. The dilutions were prepared in normal saline according to the following scale of dilutions: Dilutions from 10 to 100 at intervals of 10; from 100 to 200 at intervals of 20; from 200 to 500 at intervals of 50; from 500 to 1000 at intervals of 100; from 1000 to 50,000 at intervals of 1000.

In another series, each screw cap test tube contained a mixture of 1.5 ml saline, 0.5 ml bacterial suspension, and 0.5 ml tissue suspension, making a total volume of 2.5 ml in each tube.

All tubes were placed in a 37°C water bath containing a shaking device and kept in constant agitation for 30 minutes. At the end of 30 minutes, 2.5 ml of the first antibiotic dilution was pipetted into one of the tubes containing the mixture of saline, bacteria, and tissue fragments, making a final volume of 5 ml. The cap was screwed firmly in place and the tube shaken vigorously to wet completely the inner surface before returning it to the water bath. This same procedure was continued at 1-minute intervals for the remaining dilutions in the series. The tubes were kept in agitation in the water bath throughout the test period.

At the end of 9½ minutes, the first tube of the series was removed from the bath and the supernatant aspirated from the fragments. At the end of exactly 10 minutes, the tube was filled with about 25 ml saline to dilute the small amount of antibiotic still remaining in the tube, then set aside. The remaining tubes were treated at 1-minute intervals in the same manner. The fragments were washed 2 more times, then embedded in plasma in Carrel flasks.

The embedding medium consisted of 0.3 ml heparinized rabbit plasma, 0.5 ml Tyrode's solution, and 0.4 ml embryonic extract.* After coagulation of the plasma medium, 1 ml embryonic extract was added as nutrient. Flasks were plugged with paraffined corks and incubated at 37°C for 120 hours before making final readings.

Each flask contained a total of approximately 35 pieces of tissue measuring 0.5 to 1.0 mm in diameter. After all ingredients were added, the Carrel flasks were gently rotated to insure uniform distribution of the tissue fragments before coagulation of the plasma occurred.

Results. The highest dilution of antibiotic that showed no growth of bacteria in the Carrel flask was taken as the killing dilution. The highest dilution of antibiotic that showed no growth of tissue in the Carrel flask was taken as the killing dilution. Several series generally were necessary to determine the killing dilution for both bacteria and tissue. The toxicity index was calculated by dividing the highest dilution of antibiotic required to kill the tissue fragments by the highest dilution required to kill the test organism. Results of tests on a number of well-known germicides (1) have shown iodine to exhibit the greatest degree of germicidal efficiency with a toxicity index of 0.10. This figure indicates that under the conditions of the test iodine is 10 times more toxic to the bacteria than to the chick heart tissue fragments, a remarkable figure for a germicide. Since iodine exhibited the greatest degree of efficiency of the germicides tested, it was used as the basis of com-

* The extract was prepared by mixing 10-day-old minced chick embryos with 5 times its volume of Tyrode's solution and centrifugated at 3000 rpm for 30 minutes. The clear supernatant is the embryonic extract.

TABLE I. Highest Killing Dilution of Antibiotic for Embryonic Chick Heart Tissue and *Micrococcus pyogenes* var. *albus* and Corresponding Toxicity Index.

Antibiotic	Tissue (A)	Bacteria (B)	Toxicity index (A/B)
Iodine	1:2000	1:20,000	.10
Penicillin G sodium	1:12	1:5000	.0024
Streptomycin sulfate	1:20 growth	1:1000	<.02
Polymyxin B sulfate	1:50 growth	1:80	<.63
Terramycin	1:600	1:900	.67
Bacitracin	1:10 growth	1:10 growth	—
Chloramphenicol	1:100 growth	1:100 growth	—

parison for a number of well-known antibiotics. Of the antibiotics tested (Table I) penicillin G sodium exhibited the greatest degree of germicidal efficiency, combining high potency with low tissue toxicity. A toxicity index of 0.0024 indicated that penicillin was more than 400 times more toxic to the test bacteria than to the tissue cells, and approximately 42 times more efficient than iodine. Terramycin with an index of 0.67 was inferior to iodine as a germicide. Streptomycin sulfate was nontoxic to tissue in the highest concentration used and gave an index less than 0.02. A more concentrated solution could not be prepared without sacrificing accuracy. Likewise, Polymyxin B sulfate was nontoxic to tissue in the highest concentration used but killed the test organism in a dilution of 1:80 giving an index of less than 0.63. Chloromycetin and Bacitracin had no effect on tissue or bacteria in the concentrations used which would indicate that their actions were bacteriostatic rather than bactericidal.

Summary. A new method was proposed recently for the evaluation of germicides intended for clinical application. Iodine, with a toxicity index of 0.10 exhibited the greatest degree of germicidal efficiency. The same procedure was followed for the evaluation of a group of antibiotics. Penicillin G sodium gave an index of 0.0024 which indicated that it was more than 400 times more toxic to the test organism, *Micrococcus pyogenes* var. *albus,* than to the tissue cells, and approximately 42 times more efficient than iodine. Results on other antibiotics in order of decreasing efficiency are: Streptomycin sulfate, 0.02—; Polymyxin B sulfate, 0.63—, Terramycin, 0.67. Bacitracin and Chloramphenicol were bacteriostatic and could not be evaluated. Likewise, Aureomycin HCl, Achromycin HCl, and Erythromycin could not be evaluated; the former two precipitated from solution shortly after being prepared, and the latter failed to dissolve in saline.

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Quantitative Determination of C-Reactive Protein by Complement Fixation.*†‡ (22666)

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Interest has been growing in the measurement of serum levels of C-reactive protein as an indicator of inflammatory processes, in particular, those associated with rheumatic fever, myocardial infarction, and cancer. The original determination of this substance(1) was based on the precipitate it forms with pneumococcal C-polysaccharide. Lack of sensitivity led to an immunochemical method,

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† We wish to express our appreciation to Dr. Alfred Halpern and Schieffelin and Co. for their cooperation, to Dr. Maclyn McCarty for a specimen of pneumococcal C-polysaccharide, and to Mr. Nicholas Alonzo, Mr. Bernard Schiffer, and Mrs. Evelyn Abeshouse for their excellent technical assistance.

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the reaction between C-reactive protein antigen and specific rabbit antibody. The degree of reaction is visually estimated from the quantity of specific precipitate(2,3). More recently, estimation by complement fixation (4) and gel diffusion(5) have been reported. In this communication we describe our modifications of the complement fixation method of Muschel and Weatherwax and the theoretical and practical reasons for them. Our purpose was 2-fold: to establish an objective index of the serum level of C-reactive protein, and to control the variables inherent in the quantitative determination of antigens by complement fixation.

The method described was designed to measure C-reactive protein in the range determined in the microprecipitation method, using approximately the same quantity of antiserum, and furnishing the result within a short time.

Materials and methods. Human test sera. Test sera were inactivated at 56°C for 10 minutes(4). Specimens so treated showed no residual complement activity at a dilution of 1:10. No loss was observed in the standard CRP serum after this treatment.

Rabbit anti-CRP serum. Native antiserum preserved with sodium azide (1:1000) was obtained from Schieffelin and Co. Antibodies to normal serum components were absorbed by adding lyophilized normal human serum, 0.05 g/ml. After incubation at 40°C for 2 hours and 4°C for 2 days, a small quantity of precipitate formed. The treatment to this point was based on the procedure Schieffelin and Co. employs to make its reagent specific. Removal of the precipitate by the usual centrifugation results in an antiserum which is anticomplementary because of the presence of soluble immune complexes. This is the source of the anticomplementary property reported by Muschel and Weatherwax(4). The absorbed antiserum was centrifuged in the #40 rotor of a Model L Spinco ultracentrifuge for 2 hours at 35,000 rpm (110,000 x g). The floating lipid layer (4 mm) was rejected and the next 42 mm of fluid was withdrawn in several portions which were subsequently combined. The lowermost 12 mm of clear supernatant was then removed and kept separately. Examination of anticomplementary property showed that this increased with sample depth, but the recombined specimen described was relatively free of this interference (hemolysis greater than 55% with two 50% hemolytic units of complement at antiserum dilution 1:10). The antiserum, preserved with merthiolate (1:10,000) and stored at —20°C, is used without inactivation, since its complement is removed during the absorption. Antiserum from the lowest fifth of the tube was too anticomplementary for complement fixation tests but was useful for microprecipitation and gel diffusion analyses.

Standard CRP serum. A specimen of serum with a high CRP level was stored in small quantities at —20°C and left at 4°C after being thawed. This standard was assigned a titer of 160 after comparison with an earlier standard which had been arbitrarily set at 100. The quantity of CRP in the standard, determined by precipitation with C-polysaccharide according to the method of Wood and McCarty(3) was 280 μg/ml.

Complement fixation experiments. Reagents and procedure have been described in detail(6).

Evaluation of reactions. The results are expressed as the quantity of CRP serum (antigen) required to obtain 50% hemolysis with a constant quantity of antiserum and a constant quantity of complement. When partial hemolysis greater or less than 50% was actually measured, the quantity for this endpoint was obtained by graphic interpolation from a plot of log dilution of antigen versus log (% hemolysis ÷ 100 — % hemolysis). The routine complement fixation test was set up in 8 tubes with dilutions of CRP test serum 1:10, 1:40, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200. The quantity of complement used was four 50% hemolytic units (about 0.005 ml undiluted guinea pig serum). Antiserum was used at a dilution of 1:40 (0.00125 ml). The standard CRP serum was run with each test at dilutions of 1:800, 1:1200, 1:1600, and 1:2400. Anticomplementary property of test sera at 1:10 dilution was examined with 2 units of complement.

Incidence of AC property (hemolysis of less than 55%) at this dilution is very low (less than 1%).

Microprecipitation reaction. This test was performed in capillary tubes(3) with the following modification to insure complete mixing of reagents. 0.01 ml of antiserum was placed in the well of a clear glass spot plate. 0.01 ml of test serum was added. The entire mixture was run into the capillary tube by tipping the plate. The contents of the capillary were then almost completely returned to the spot plate well by gentle (mouth) pressure. This procedure was repeated and the capillary tube was sealed after the third filling. Tubes were read by comparison against the standard CRP serum (equal to 160) at concentrations of 80, 40, 20, 10, 5 and 2½ after incubation (2 hours at 37°C, overnight at 4°C). With the antiserum used, the supernatant from the "80" tube produced no additional precipitate on adding either antiserum or CRP serum. The quantity of precipitate in the "2½" tube was less than 1 mm but more than a trace.

Gel diffusion. This test was carried out by the modification of the Ouchterlony technic described by Korngold and Lipari(7) which uses 1.5% concentration of agar. Plates were kept at room temperature for one week and then, as described by Libretti, *et al.*(5), at 4°C for 2 weeks.

Results. The complement fixation test for CRP involves 3 main variables—namely, the quantities of antiserum and complement which are required for the most effective measurement of a given quantity of CRP. The interrelationships are best studied by means of a block titration in which the quantity of complement is held constant and antiserum and antigen are varied. The data can be integrated into a line curve expressing the relationship between antigen and antibody over the full range of antibody excess, equivalence, and antigen excess by plotting the quantities of these reactants which always produce the same hemolytic endpoint(8). The relationship for the rabbit antibody-CRP system is shown in Fig. 1a. The curve is a non-rectangular hyperbola with asymptotes in the zones of antibody excess and antigen excess and a minimum close to the zone of equivalence. This curve is representative of systems in which the zone of antigen excess is characterized by marked inhibition of immune complex formation(8). It can be seen that for the quantity of complement with which the curve is determined, a minimum quantity of CRP can be measured, no matter how large an excess of antibody is used. In Fig. 1b there is shown on expanded axes the zone of antibody excess determined with each of 3 different quantities of complement: 3, 4, and 5 units. These curves show how sensitivity of the determination (minimum quantity of measurable CRP) depends on quantity of complement used in the test, and further, how much antiserum must be used to insure antibody excess, once the sensitivity level has been selected. The choice of a particular set of quantities is a balance between supply of antiserum on one hand, and on the other, both quantity of CRP which must be determined and degree of interference from anticomplementary properties of the reactants.

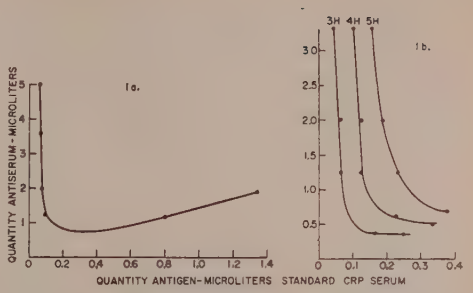

FIG. 1a. Antigen-antiserum reactivity curve for human CRP-rabbit antibody system.

FIG. 1b. Curves in zones of antibody excess and equivalence determined with 3, 4, and 5 hemolytic units of complement.

A correlation of CRP titers determined for 192 specimens by both complement fixation and precipitation methods is shown in Fig. 2. It is evident that sensitivity of the method based on complement fixation has been set to conform exactly to sensitivity of that based on microprecipitation. Twenty-four specimens gave zero titers with both methods, 8 showed a CF titer of 1 without producing any precipitate, while 4 showed a precipitate corresponding to 1, 2 a precipitate correspond-

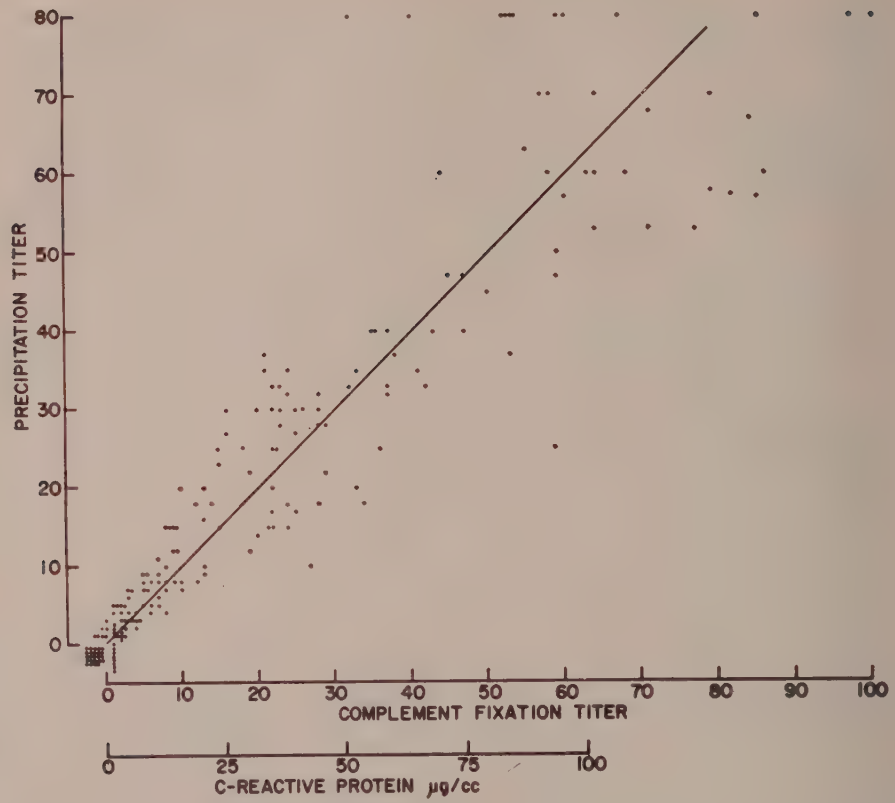

FIG. 2. Correlation of CRP levels determined by microprecipitation and complement fixation showing identical range of both methods. Specimens with very high complement fixation titers are not shown since maximum precipitation titer obtainable with undiluted test specimens is "80." Precipitation titers are the avg of observations by 3 individuals. The line represents the location of points for exact correspondence.

ing to 2, and 1 a precipitate corresponding to 3 without having any CRP determined by complement fixation. If we accept the greater reliability of the complement fixation method (since it is applied at a level considerably above the limit of its sensitivity), these results suggest that very small quantities of precipitate may not be specific for CRP. It has been frequently noted that traces of precipitate tend to redissolve on standing several minutes at room temperature. The relatively poor correlation between CRP titers obtained by the two methods is principally due to the error inherent in the subjective (visual) method of estimating the quantity of precipitate. Distinguishing a titer of "40" from one of "20" or "80" is frequently impossible, and the degree of correlation shown in Fig. 2 could be achieved only by averaging the estimates of 3 observers. The complement fixation method, on the other hand, offers an objective numerical index of the CRP titer. If 2-fold serial dilutions are tested and the exact dilution titer for 50% hemolysis is estimated by the graphical method described, an error of as much as 10% hemolysis produces an error of about 10% in the final result. Closer spacing of dilutions, as described for the standard, can appreciably decrease this source of variation.

The hemolytic unit of complement (quantity of guinea pig serum) which is defined by the indicator reaction serves as a control of the reagents participating in this part of the

test. Fluctuations in the unit result principally from variations in batches of sensitized red cells and guinea pig serum. However, this index of variability in reagents is not applicable to the primary reaction. For this purpose, we have found that the use of a standard is essential(6). A convenient stable standard is a high-titered CRP serum specimen. The data in Table I representing dilution titers of the standard serum determined over a 5 month period and complement unit on the day of each determination show that the standard is stable. They also indicate that with a higher complement unit, a lower dilution titer is observed. This explanation is not sufficient to account for the variation or to eliminate the necessity for the standard, as is seen by inspecting the values on 1/11, 1/18, 1/27 and 1/31. On these days the complement unit was constant but the standard changed 35 to 45%.

The quantity of CRP in the standard was 280 μg/ml. Average titer was about 1500. Since 0.1 ml of antigen dilution is used, the endpoint in the complement fixation test requires about 0.02 μg of C-reactive protein. The sensitivity of the test described here (with limiting dilution of 1:10) therefore corresponds to 2 μg CRP/ml undiluted serum, a result in excellent agreement with the report of Wood and McCarty‡(3). An example of the calculation of CRP titer or concentration based on the standard is as follows. Assuming the test specimen shows a dilution titer of 350 when the standard serum dilution titer is 1500, then

$$\text{Test specimen CRP titer is } \frac{350}{1500} \times 160 = 37.$$

$$\text{Test specimen CRP conc. is } \frac{350}{1500} \times 280\ \mu\text{g/ml} = 65\ \mu\text{g/ml}.$$

In gel diffusion studies by the method of Ouchterlony we have not observed the multiplicity of CRP components reported by Libretti, *et al.*(5), either with 20 high titered specimens from different individuals or a pool of 20 such specimens. On prolonged standing (more than 10 days), a pattern similar to the one they describe was seen, but this is an artifact of the diffusion process, particularly in the region of antibody excess(9).

‡ The values for concentration of CRP in Table 4 of this reference should be divided by 2 according to the authors.

TABLE I. Titers of Standard CRP Serum and Complement over 5 Month Period Showing Stability of Standard and Relative Independence of Fixability and Hemolytic Activities of Guinea Pig Complement.

Date	Dilution titer of standard CRP serum	Complement unit, ml g.p. serum
9/12	1000	.00142
11/ 1	1800	.00122
11/ 9	1750	.00110
11/16	1450	.00110
11/30	1300	.00110
12/ 7	1750	.00121
12/14	1700	.00110
12/21	1150	.00121
12/28	1450	.00105
1/ 5	1400	.00100
1/11	1900	.00105
1/18	1350	.00105
1/27	1750	.00107
1/31	1300	.00105
2/ 8	1250	.00122

Discussion. In the procedure herein described, we have paid particular attention to 3 aspects of quantitative immunochemical analysis by complement fixation. These are the removal of residual anticomplementary activity from absorbed antisera, the necessity for a standard to eliminate variations as large as 2-fold in "fixability" of complement, and the selection of the most suitable quantity of antiserum for a given purpose. The range of C-reactive protein levels usually found in human serum is 0 to 300 μg per ml(3). The level can rise well above 300 μg after surgery (10). The large range taken in conjunction with the observed inhibition by excess antigen determines minimum number of tubes which must be set up for the complement fixation test. A 10 to 20-fold excess of antigen over the quantity measured at the endpoint is sufficient to produce hemolysis indicating a weak or negative test; the inhibition produced by a lesser degree of antigen excess may still appreciably affect determination of titer.‖ Suitable modifications of the complement fixation

‖ Since titer is usually determined from hemolysis greater or less than 50% by interpolation, a partial hemolysis in the zone of inhibition will incorrectly reduce the titer.

method will permit measurement of considerably lower concentrations of C-reactive protein than 2 μg per ml. However, as sensitivity is increased, two limitations become progressively more important: incidence of anticomplementary activity in test specimens, and C-reactive protein interaction with normal serum constituents which affects its reaction with antibody.

Summary. A quantitative immunochemical method for determination of C-reactive protein concentration in serum is described. The sensitivity of this method, based on complement fixation, has been selected to measure the same concentration range of CRP as determined by microprecipitation. Two important features generally applicable to complement fixation methods are presented; namely, removal of anticomplementary activity from absorbed antisera and use of a reference standard to compensate for variations in fixability of complement.

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Pheochromocytomas of Adrenals in Male Rats Chronically Injected with Pituitary Growth Hormone.* (22667)

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Increased numbers of tumors of the types occurring spontaneously in the rat were observed during the course of chronic injection of normal female rats with pituitary growth hormone. Pulmonary and lymphatic tissues, reproductive organs, pituitary and adrenal glands were involved(1-6). Outstanding was the occurrence of adrenal medullary pheochromocytomas(2). Since none of this work had hitherto been done in male rats, the questions arose as to whether males would be equally responsive in body growth and similarly susceptible to tumor formation, especially as regards reproductive organs and adrenal glands.

Materials and methods. Male rats of the Long-Evans strain, of 2 different age groups, 6 and 14 months, were divided into experimental and control groups of 8 rats each. The purified growth hormone(7) was injected intraperitoneally in saline 6 days weekly. The initial daily dose of 0.5 mg was increased progressively during the first 160 days, reaching a maximum of 3.0 mg in the younger, and 3.5 mg in the older group. These doses were then maintained for the remainder of the 15-month injection period. Complete autopsies were performed on all rats. The weights of all organs were determined, and the entire organ or representative blocks were fixed and prepared for histological study.

Results. Body weight and length. The rats in the 6-month-old group weighed approximately 400 g at the beginning of the experiment and gained steadily, reaching a maximum of almost 700 g at 18 months (Fig.

* Aided by grants from the United States Public Health Services A-366 and C-1098; and The University of California Cancer Research Coordinating Committee.

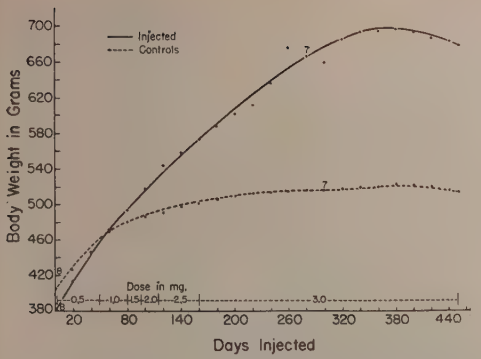

FIG. 1. Avg body wt of male rats inj. with growth hormone for 15 mo, beginning at 6 mo of age, compared with uninj. controls. Dosage shown in scale above abscissa. (No. of rats on which avg is based is indicated by numbers above curves.)

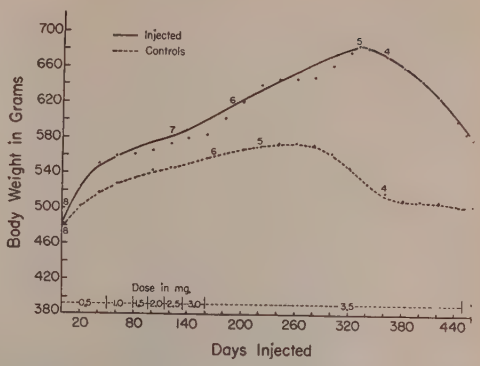

FIG. 2. Avg body wt of male rats inj. with growth hormone for 15 mo, beginning at 14 mo of age, compared with uninj. controls.

1). During the last 90 days there was cessation of growth and some loss of weight, despite the high daily dose. The rats of the 14-month group (Fig. 2) were heavier at onset (480 g) and their gain was slower and less regular, the maximum weight attained during the 15-month period being slightly less than that of the younger group, even though they received a higher dose. Both experimental groups increased in total length above their controls, and the greater increase occurred in the younger group.

Blood pressure. Because of the anticipated development of pheochromocytoma, a neoplasm which had occurred frequently in female rats similarly treated(3), determinations of blood pressure were made at intervals during the course of this experiment. A microphonic manometer for determination of the systolic blood pressure was used(8). The animals were anesthetized with ether for determination of blood pressure, and body length was determined at the same time. No significant differences were observed in the blood pressure of experimental and control rats.

TABLE I. Neoplasms in Growth Hormone Injected Male Rats and Controls (8 per Group).

Type of neoplastic lesion	Age at onset of exp. 6 mo Inj.	6 mo Controls	14 mo Inj.	14 mo Controls
Pheochromocytoma	4	0	5*	0
Adrenal cortical adenoma	0	1	0	1
Thyroid: Adenoma	3	4	3	1
Carcinoma	1	0	1	1
Pituitary, adenoma	4	6	4	5
Testis, Leydig-cell tumor	1	0	0	0
Thymus, squamous cell carcinoma	0	1	0	0
Lymphosarcoma, thoracic and abdominal	1	0	4	3
Mesenchymal sarcoma (synovioma)	1	0	0	0
Kidney, papillary cystadenoma	0	0	1	0

* Bilateral in 4.

Neoplastic lesions. Neoplasms occurred in lymphoid tissue, adrenals, pituitary and thyroid glands. There was nothing significant in regard to the reproductive system. The number of tumors in the growth hormone injected rats of the 2 ages was not significantly different from that in the controls,† except in the tumors of the adrenal medulla. Neoplasms of the adrenal medulla were the most outstanding lesions observed in the experimental animals. Even in the adrenals where no pheochromocytomas were present, there was enlargement of the medullas. None of the controls of either age group had pheochromocytomas; 3 had focal areas in the medulla composed of uniformly small cells adjacent to blood vessels. Pheochromocytomas were

† Numbers of tumors exclusive of pheochromocytomas, in the 6 months old group, 11 in experimental, 12 in control; in the 14 months old group, 13 in experimental, 11 in control.

present in 9 of the 16 rats treated with growth hormone; multicentric foci were present in some (Fig. 3-5). In the younger group, 4 of 8 rats showed unilateral tumors. In the older

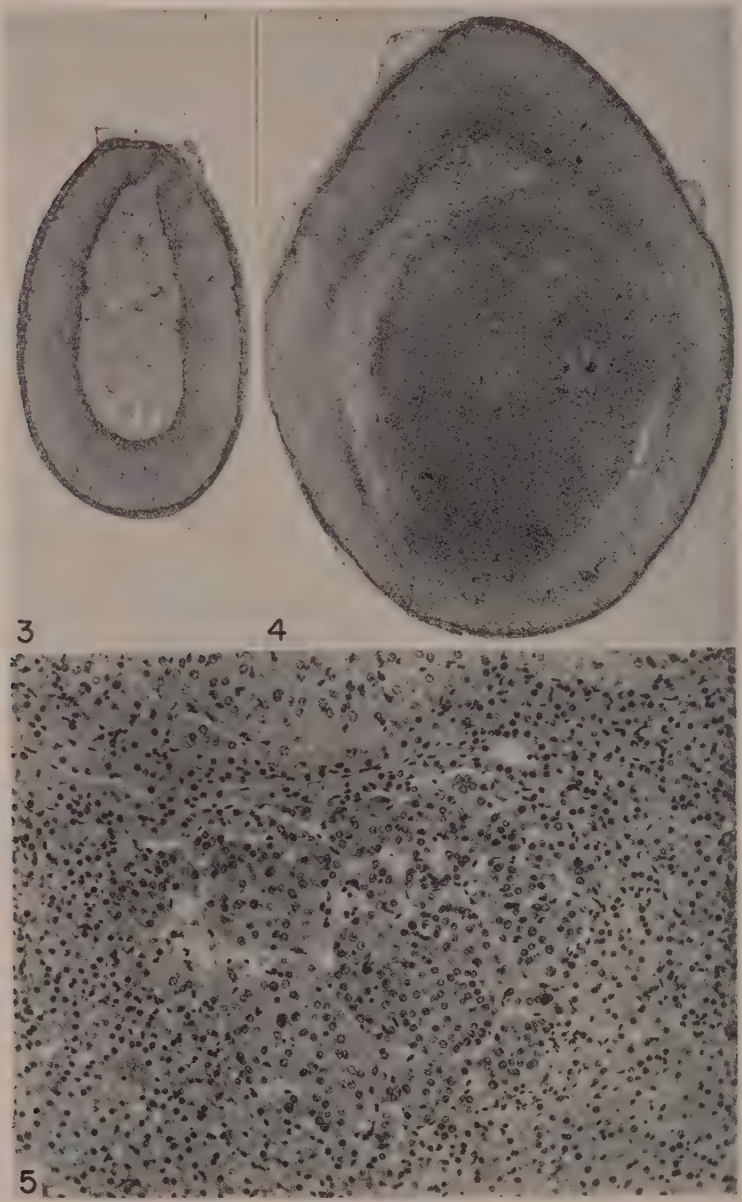

PLATE I (Fig. 3-5). Adrenals of adult male rats, control and exp. (H and E stain)

FIG. 3. Control, untreated. Age 21 mo. × 22.

FIG. 4. Large medullary pheochromocytoma. Note compression of normal medullary tissue. Growth hormone inj. begun at 6 mo of age. × 22.

FIG. 5. Small pheochromocytoma composed of large cell types; surrounded by cells of zona reticularis. Growth hormone inj. begun at 14 mo of age. × 250.

group there were lesions in 5 of 8 rats and in 4 instances these were bilateral. Invasion of blood vessels by neoplastic cells, as described by Gillman *et al.*(9) in spontaneous pheochromocytomas of the rat, was not observed.

Discussion. Analysis of the results of chronic administration of pituitary growth hormone to adult male rats indicates that growth response was similar to, but less striking than, that of females so treated. Growth response of the older of the 2 age groups tested (14 months old at onset) was inferior to that of the younger group (6 months old at onset), particularly in the later part of the experiment. This may have been due to a number of factors, such as aging, debility due to neoplasms, and recurrent infections. In the earlier studies on females injected with growth hormone, pheochromocytomas frequently occurred. There was no evidence of a prolonged hypertension in those animals, as judged by weight of the heart and histology of the arterioles. In the present study no significant elevations of blood pressure were observed in experimental rats subsequently shown to have pheochromocytomas. However, the possibility of transient episodes of hypertension cannot be excluded.

Summary. The long term administration of pituitary growth hormone to adult male rats of the Long-Evans strain resulted in progressive growth in body weight and length. Many neoplasms developed in the rats during this prolonged period, but the incidence in treated rats was not significantly different from that in controls, with the exception of tumors of the adrenal medulla. Pheochromocytomas occurred in 9 of 16 growth hormone injected rats and in none of the controls.

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Influence of Acetylcholine on Human Pulmonary Circulation under Normal and Hypoxic Conditions.* (22668)

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Short periods of induced hypoxia characteristically produce a moderate increase in the pressure in the pulmonary artery in normal man(1). In general, the rise in pressure is greater than might be expected to be due to the increase in cardiac output which occurs. Moreover, the possibility that the augmented pressure results from generalized systemic vasoconstriction seems unlikely, since the pulmonary wedge pressure does not change(2,3) the brachial artery pressure remains constant, and the "central blood volume" shows no consistent alteration(3,4). From these data it may be inferred that the

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† Fellow of the Nuffield Foundation.

‡ Fellow of the New York Heart Association.

§ Postdoctoral Research Fellow, Public Health Service.

rise in pulmonary artery pressure is probably produced by active constriction of the vessels of the lungs.

Previous experiments(5,6) have led us to believe that acetylcholine has a vasodilating action in the human pulmonary circulation. In diseased states there is evidence that this action is to some extent dependent on the pre-existing tone in the vessels of the lungs(5,7). The effect of acetylcholine might, therefore, be expected to be more evident when the tone in these vessels has been increased by hypoxia.

The present study was undertaken to investigate the effect of acetylcholine on normal pulmonary circulation, and to determine whether the effect was modified by the presence of hypoxia. The purpose of this note is to report the results obtained in studies performed on 6 normal subjects.

Methods. The procedure combined catheterization of the pulmonary artery and cannulation of the brachial artery. Each subject breathed through a mouthpiece for 2 periods of 20 minutes separated by a rest of 15 minutes. During one period, the subject breathed ambient air and, during the other, a mixture of 12% oxygen in nitrogen. Between the 11th and 15th minute of each period, acetylcholine was infused through the catheter into the main pulmonary artery at a rate of 0.5 mg/min. Measurements of the pressure in the pulmonary and brachial arteries and of the cardiac output were made before and during the infusion of acetylcholine.

While the subject breathed ambient air, the cardiac output was estimated by the Fick principle. Under hypoxia, the Stewart-Hamilton dye-dilution technic was considered preferable since insufficient time was allowed for the establishment of the "steady state" required for the application of the direct Fick method. The dye output was obtained by injecting Evans blue dye into the main pulmonary artery while inscribing a dilution curve from the brachial artery by means of a Colson densitometer.

Results. Six satisfactory studies have been completed with similar results in each. The data from one of these are illustrated in Fig. 1. When the subjects breathed ambient air, acetylcholine caused a slight fall in

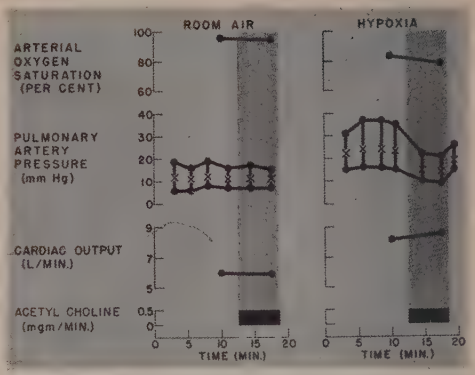

FIG. 1. Effect of acetylcholine on arterial oxygen saturation, pulmonary arterial pressure and cardiac output in one subject. The subject breathed room air for 20 min. and 12% oxygen for 20 min. Shaded areas represent time of infusion of acetylcholine. A period of 15 min. elapsed between the 2 experiments.

the pulmonary arterial pressure. This response was much more evident after hypoxia had raised the pressure in the pulmonary artery. The reduction in pressure during the infusion of the drug occurred despite the fact that the cardiac output had either remained constant or had slightly increased. The infusion caused no alteration in the heart rate, the brachial arterial pressure, the arterial oxygen saturation, or the ventilation. In 2 subjects, pulmonary wedge pressures were recorded and found not to change during the infusion of acetylcholine. Thus, since the pressure in the pulmonary artery had fallen, the gradient of pressure was decreased.

Discussion. In these studies, the cardiac output remained unchanged or increased during the infusion of acetylcholine; it never decreased. Moreover, the constancy of the wedge pressure measured in 2 cases suggests that the left atrial pressure was unaffected by the drug. Therefore, it is believed that the fall in pulmonary arterial pressure resulted from an active dilatation of the pulmonary vessels. The augmented effect observed during hypoxia suggests that the action of the drug is dependent on the pre-existing tone of the pulmonary vessels.

Summary. Acetylcholine infused into the main pulmonary artery caused a slight fall in pulmonary arterial pressure when the subject

breathed ambient air, but a greater fall in pressure after pulmonary hypertension had been produced by hypoxia. The fall in pressure was associated with either a constant or an increased cardiac output. The evidence suggests that acetylcholine causes vasodilatation in the lungs and that this action is largely dependent on the pre-existing tone of the pulmonary vessels.

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Relationship of Bat Salivary Gland Virus to St. Louis Encephalitis Group of Viruses.* (22669)

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The existence of rabies in bats in the United States was first suspected in 1951 when a woman in Big Spring, Texas died of rabies 3 weeks after being bitten by a bat. This experience was reported(1) after the first demonstration of rabies in bats in the United States when the virus was demonstrated in a Florida yellow bat (*Dasypterus floridanus*) which had attacked a child(2). Three months after this incident another case was reported in Pennsylvania where, without provocation, a woman was bitten on the arm by a bat (probably *Lasciurus cinereus*) from which the rabies virus was isolated(3,4). These and other experiences prompted several groups to initiate studies to assess the importance of these animals as reservoirs and carriers of the infection(5-10). The rabies virus has now been recovered from bats in widely scattered areas in the United States (Florida (2), Pennsylvania(3), Texas(6,7), California (10), and Montana(11).)

During the course of their studies on rabies in nonsanguivorous bats in Texas, Burns and Farinacci(8,12,13) recovered a number of viral agents in addition to the rabies virus from the salivary glands of encephalitic bats. Following preliminary tests(13) which suggested that these agents were antigenically related to the St. Louis encephalitis virus, one strain was referred to us for determination of its antigenic relationship to the Japanese (JE)-West Nile (WN)-St. Louis (SLE)-Murray Valley (MVE) encephalitis group(14-19). This report is concerned with the relationship of the bat salivary gland (SG) virus to the JE-WN-SLE-MVE group as established by cross complement-fixation tests using hyperimmune guinea pig sera.

Viruses. The bat salivary gland virus (SG) designated #1410-19 and in its 5th mouse brain passage was sent to us by Lt. Col. Kenneth F. Burns. The Nakayama strain of JE virus in the 41st mouse brain passage, a strain of MVE in the 10th passage, and the WN virus (J7259) in the 25th passage were obtained from the Viral and Rickettsial Disease Registry, American Type Culture Collection, Washington, D.C. The Hubbard strain of SLE was obtained from Dr. Isaac Rushman.

Antigens and antisera. The antigens and the antisera for the complement fixation tests and the grid type test using these materials are essentially the same as described by Pond and his associates(19) and by Hammon and Espana(20). Antisera against the JE, WN, SLE and MVE viruses were obtained from

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guinea pigs inoculated intracerebrally with suspensions of infected hamster brain. In each instance the virus contained in infected mouse brain tissue in 10% normal rabbit serum was passed intracerebrally through 3 serial passages in weaned hamsters. The guinea pigs were given 2 injections, 10 days apart, each consisting of 0.15 ml of a 10% suspension of 3rd passage infected hamster brain tissue. The sera obtained 2 weeks after the last injection were stored at —40°C. Each serum was inactivated at 56°C for 30 minutes before it was used in the complement fixation test. Since the SG virus is not infective for young hamsters, antiserum against this agent was prepared as described above, except 6th mouse brain passage material in 10% normal guinea pig serum in saline was used for immunizing the guinea pigs. A control group of guinea pigs each received two injections of 10% normal mouse brain in 10% guinea pig serum in saline.

In the initial tests it was found that all of the viruses except SG yielded effective complement-fixing antigens prepared by the technic of Havens *et al.*(21) or Casals *et al.*(22). Since highly effective antigens with the SG virus were obtained by the benzene extraction technic of DeBoer and Cox(23), all complement fixation tests were carried out with antigens prepared by this method.

Complement fixation tests. The complement fixation tests used in these studies are essentially the same as those described by Pond *et al.*(19). Serial 2-fold dilutions of each antigen were tested against serial 2-fold dilutions of each of the guinea pig antisera. In addition to the usual serum and antigen anti-complementary controls, normal guinea pig serum in serial dilution was tested against each dilution of the various antigens. In the test 2 exact units of complement were used. Following overnight incubation at 4°C the indicator system consisting of 3 units of hemolysin and 3% washed sheep cells was added. The tests were read after an additional incubation for 30 minutes at 37°C. Tubes with 50% or more fixation were considered positive.

Results. The results of a grid-type complement fixation test are presented graphically in Fig. 1 using the method previously described by Pond *et al.*(19). The shaded areas indicate mixtures in which 50% or more fixation of complement occurred. The results with the JE, MVE, WN and SLE antigens are in some respects the same as described by Pond *et al.*(19), although the higher titered SLE antigen used in these tests demonstrated serological relationships with other members of the family which were not evident in their study. For example, it can be seen that the JE antiserum reacted with its homologous antigen and to some extent with the SLE antigen, but failed to react with the MVE antigen. Pond *et al.*(19), however, found that JE antiserum reacted with the MVE antigen but not with the SLE antigen. In the results described in Fig. 1 it can be seen that the SLE antigen reacted to some extent with each of the antisera tested.

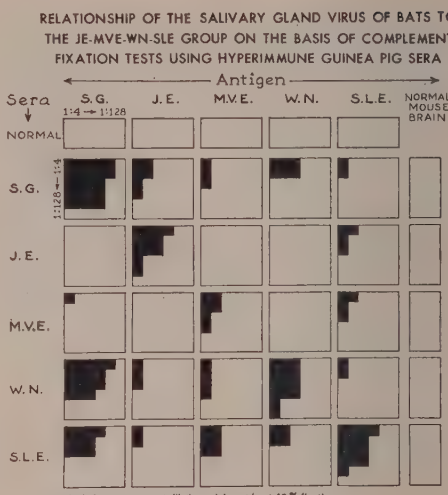

FIG. 1.

The results indicate that the SG agent appears to be a new member of the JE-WN-SLE-MVE group of viruses and that it is more closely related antigenically to WN than to other members of the group. These data, together with the fact that the SG antigen failed to fix complement in the presence of antisera prepared against a variety of other neurotropic viruses, confirm the preliminary

observations of Burns and Farinacci(13). Additional studies are in progress to establish whether the serological relationships observed in the complement fixation test can be more effectively demonstrated by other immunologic technics.

Summary. A virus recently isolated from the salivary glands of Mexican free-tailed bats (*Tadarida mexicana*) appears to be a new member of the JE-WN-SLE-MVE group of viruses. On the basis of the results of complement fixation tests it appears that the salivary gland virus of bats is more closely related to the West Nile virus than to other members of the group.

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Choline Antimetabolites. Studies with Intestinal Muscle. (22670)

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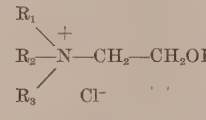
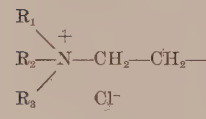
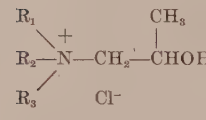
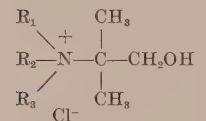
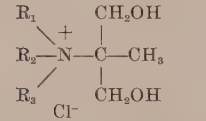
The report of Keston and Wortis(1) that frog muscle contracts in response to choline and that this contraction is inhibited by triethylcholine was of interest to us in our studies of compounds structurally similar to choline. Triethylcholine is only a very weak inhibitor of choline oxidation by choline dehydrogenase(2) and has usually been found to have choline-like activity (*Neurospora crassa* growth(3); lipotropic(4); antihemorrhagic (5)). In view of this anomalous behavior of triethylcholine with muscle we have extended our studies to include the effects of choline and related compounds on muscle contraction.

In this study segments of rabbit ileum were used instead of frog muscle. This acetylcholine sensitive tissue contracts in response to choline. We found that the response to choline was inhibited competitively by triethylcholine, β-methyltriethylcholine and α-methyl-α-hydroxymethylcholine. Triethylcholine was not, however, an inhibitor of acetylcholine. Methyldiethylcholine, triethylhomocholine, and α-methyl-α-hydroxymethyltrie-

TABLE I. Effect of Choline Analogues on Rabbit Ileum Muscle.

Compounds tested	R_1	R_2	R_3	Choline-like (relative activity, choline = 1.0)	Anticholine
	H	H	H	.3*	
	E	E	H	.1	
	M	M	E	4.0	
	M	E	E	.0	—
	E	E	E	.0	+
Ethanolamine					
	H	H	H	.2	
	E	E	H	.1*	
	E	E	E	.0	—
	M	E	E	.1	
3-Aminopropanol-1					
	E	E	H	.1*	
	M	E	E	.1*	
	M	M	M	.5	
	E	E	E	.0	+
1-Aminopropanol-2					
	H	H	H	.1*	
	M	M	M	.2	
	E	E	E	.1	
2-Amino-2-methylpropanol-1					
	H	H	H	.1*	
	M	M	M	.0	+
	E	E	E	.0	—
2-Amino-2-methylpropanediol-1,3					
Thiocholine chloride				3.1	

* Transient response. M = Methyl; E = Ethyl.

thylcholine were inactive at the concentrations tested while the remaining compounds studied possessed various degrees of choline-like activity.

Methods and materials. The choline analogues were prepared by methods previously described(2). Rabbit ileum was obtained soon after sacrifice of the animal, washed with Tyrode solution and then immersed in this medium at 5°C until used. A small segment was suspended in 100 ml of Tyrode solution at 37°, through which was bubbled a mixture of 95% O_2 + 5% CO_2, and its contractions recorded with a frontal writing lever on a kymograph.

When the spontaneous contractions had become constant, 2 mg of choline chloride were added and the increased contractions recorded. The bathing medium was then removed; the segment was washed 3 times with 50 ml of fresh Tyrode solution (37°), and resuspended in Tyrode solution as above. These operations could be repeated several times with essentially the same response to choline; the response to choline chloride was proportional to the dose up to a level of 6 mg.

The effects of the choline analogues on the segments of ileum were determined by adding the former to the bathing medium 2 mg at a time until 6 mg had been used. The testing of each compound was preceded by a test with choline, which was then washed out as above, and the segment was discarded when its response to choline was appreciably decreased.

Those compounds which failed to cause an increase in the contraction of the segment were tested for anticholine activity. Two mg of choline chloride were added to the bathing medium, and after the increase in contraction was established, 6 mg of the test compound were added. If no change in the contractions was produced, the test compound was considered to have neither choline-like nor anticholine activity.

Results. The activities of the compounds tested are presented in Table I. Fourteen of the compounds produced a response like that given by choline; the potencies were usually less than 0.5 that of choline on a weight basis with the exception of dimethylethylcholine and thiocholine which were 4.0 and 3.1 times as active as choline respectively. Diethylmethylcholine, triethylhomocholine and α-methyl- α-hydroxymethyltriethylcholine were inactive at the concentrations tested while triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine possessed anticholine activities.

That the compounds having anticholine activities were actually competitive inhibitors of choline was established by determining response of the segments of ileum to choline in the presence of 15 mg of the inhibitory compounds. The results are plotted in Fig. 1. In each case the response curve was roughly parallel to that obtained with choline alone and displaced toward higher choline concentrations. Under the same conditions, however, these compounds were found to have choline-like activities with segments of jejunum.

Discussion. The ability of choline to cause contraction of intestinal muscle may be (a) an inherent property of the compound or such activity may result from (b) its conversion to acetylcholine or (c) its being an inhibitor of

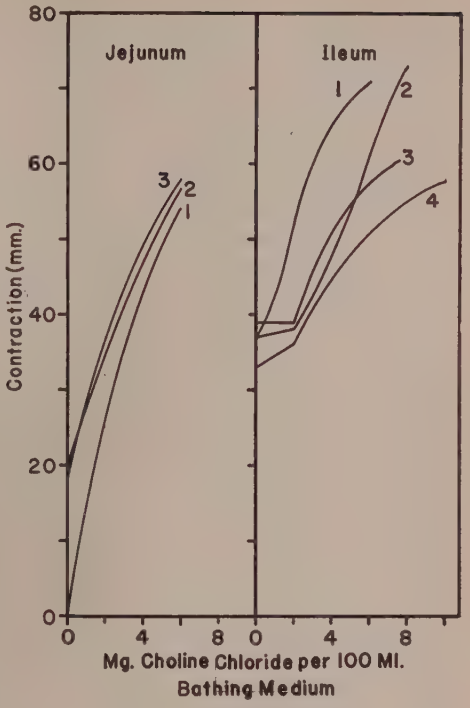


FIG. 1. Curve 1, choline alone; curve 2, choline plus 15 mg triethylcholine chloride; curve 3, choline plus 15 mg β-methyltriethylcholine chloride; curve 4, choline plus 15 mg α-methyl-α-hydroxymethylcholine chloride. In the segments of jejunum the contraction recorded was largely due to a change in tone. The contractions plotted for both tissues represent the sum of actual contraction and increased tone. Curves plotted are avg obtained with the same intestinal segment. Pieces of intestine from other animals gave similar results.

choline esterase. Whatever the mechanism of action is, it is reasonable to assume that the remaining compounds having choline-like activity would exert their activities by a similar means. That choline is a competitive inhibitor of choline esterase has been demonstrated (6,7). However, this does not seem to be the mechanism involved here since the anticholine effects of triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine would not then be explicable.

An unequivocal differentiation between the first two possible modes of action cannot be made from the experiments done in this study. However, there is some evidence against the second interpretation. First, we ob-

served that the contractions of the ileum began almost immediately on the addition of the choline and were constant after a relatively few contractions. If it were necessary to acetylate the choline in order for it to be active one might expect a delay followed by a relatively long period of increasing contractions. Second, by analogy to studies of the action of choline and related compounds on the heart of *Venus mercenaria*(8, 9) it seems also that the first possibility is more likely. It thus appears that our results are best explained by assuming that choline and certain other compounds related to it are capable *per se* of stimulating intestinal muscle. The mechanism of this action may be similar to the coenzyme theory proposed by Welsh(10) to explain the mode of action of acetylcholine. Accordingly, triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine have anticholine activity because they compete with choline for active sites on the apoenzyme.

Summary. Choline-like and anticholine activities of a series of compounds structurally related to choline have been determined using segments of rabbit ileum. Triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine were competitive inhibitors of choline in this tissue but had choline-like activity on segments of jejunum.

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Effect of Aspirin Administration on Serum Glutamic Oxaloacetic and Glutamic Pyruvic Transaminases in Children.* (22671)

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(Introduced by H. F. Wood)
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Elevations of the serum glutamic oxaloacetic transaminase (SGO-T) activity during the course of rheumatic fever in children have been noted previously(1). Despite the irregular pattern of these elevations and the absence of parallel alterations in the indices of inflammation commonly used (erythrocyte sedimentation rate, white blood cell count and C-reactive protein) and in the clinical course of the patients, it was suggested that the alterations of SGO-T were the result of myocardial necrosis during the course of rheumatic carditis. This enzyme is known to be present in the heart in high concentration(2) and elevation of its activity in the serum following myocardial infarction in man(3) and in dogs (4) has been described. Elevations of SGO-T also occur in human(5) and murine(6) hepatic diseases, but no clinical evidence of hepatic disease was present in the rheumatic fever patients studied and their liver function tests were normal(1). Drugs such as digitalis and mercurial diuretics which are often used in the treatment of patients with rheumatic fever were shown to have no effect on

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the SGO-T(3). Moreover, no alteration of SGO-T was noted during the administration of 4 to 6 g of aspirin daily for one week to a group of non-rheumatic adults(1).

Despite this latter observation a possible effect of aspirin upon the serum activity of glutamic oxaloacetic transaminase in children was suspected as a larger series of children with rheumatic fever was subsequently studied. The present investigation was undertaken to explore this relationship. The activity of another enzyme, glutamic pyruvic transaminase (SGP-T) was studied in the sera of a group of patients in an attempt to determine whether serum activity of another transaminase in addition to SGO-T was affected by aspirin administration. In addition, since glutamic pyruvic transaminase is found in the liver in 6 times the concentration found in the heart(7), it was hoped that the source of the increased serum concentration of SGO-T might be suggested by the results of simultaneous determinations of the two transaminases. It has, in fact, been reported, in contrast to the findings with SGO-T, that the serum activity of glutamic pyruvic transaminase (SGP-T) is increased only slightly following myocardial necrosis in man. Similar elevations of the serum activities of both enzymes are found in liver diseases(7).

Materials and methods. Twenty-three children from the wards of Irvington House, a hospital and convalescent home for children with rheumatic fever, were studied. Their ages ranged from 5 to 15.

Group 1: Of the 14 children included in this group 12 received aspirin (acetyl salicylic acid) and 2 sodium salicylate. None was considered to have definite evidence of active rheumatic fever: 7 had questionable disease activity and 7 had no evidence of rheumatic activity by all clinical and laboratory criteria. Aspirin and sodium salicylate were administered in daily dosages of 0.6 to 1 g per 15 lb of body weight for a minimum of 4 weeks. The SGO-T was measured during a short control period and then twice weekly during the period of salicylate administration.

Group 2: The second group selected for study of both SGO-T and SGP-T consisted of 9 children, 7 of whom had no clinical or laboratory evidence of rheumatic activity. One patient was studied during a mild clinical rebound of rheumatic fever. The ninth patient had acute rheumatic polyarthritis with no evidence of carditis. Aspirin was administered in a daily dose of 0.6 g per 15 lb of body weight for an average period of 16 days. The dose was then increased to 1 g per 15 lb of body weight until symptoms and signs of toxicity occurred. At this time the aspirin was discontinued.

The SGO-T and SGP-T activities were determined twice weekly during a short control period and during the period of aspirin administration. The specimens of blood obtained were allowed to clot at room temperature. The sera were then separated immediately and frozen and the determinations of enzyme activity were performed within 3 days. Care was exercised to prevent hemolysis since this has been shown to increase the measured SGO-T activity(8). SGO-T activity was measured by the spectrophotometric method of Karmen *et al.*(8). SGP-T activity was measured by a similar method described by Wróblewski and LaDue(7). By these methods the normal range of activity of SGO-T is 10-40 u/ml/min. and of SGP-T is 5-35 u/ml/min. Rises of SGO-T to 50 units or more and of SGP-T to 45 units or higher are considered to be significant.

Blood salicylate levels were determined twice weekly using the method of Brodie, Udenfriend and Coburn(9). C-reactive protein was determined weekly by the capillary precipitin method described by Anderson and McCarty(10). Erythrocyte sedimentation rate was determined weekly by the Wintrobe method(11). To rule out the possibility of a direct effect of aspirin upon the rate of change of optical density of the reaction mixture in the spectrophotometric methods used, 2 series of experiments were done: A. Solutions of aspirin in phosphate buffer (pH 7.4) were added to the reduced coenzyme I to final concentrations of 10 to 60 mg % and the optical densities of the solutions measured for 10 minutes. B. Both sodium salicylate and aspirin were dissolved in normal serum to

TABLE I. Effect of Aspirin and Sodium Salicylate Administration on Serum Glutamic Oxaloacetic Transaminase Activity.

Patient	Rheumatic activity	Drug administered	Maximal level of SGO-T (u/ml)
1	?	Aspirin	146
2	?	"	88
3	?	"	128
4	No	"	93
5	?	"	<50
6	No	"	132
7	"	"	70
8	"	"	<50
9	"	"	"
10	?	"	"
11	?	"	"
12	No	"	"
13	"	Sodium salicylate	100
14	?	" "	<50

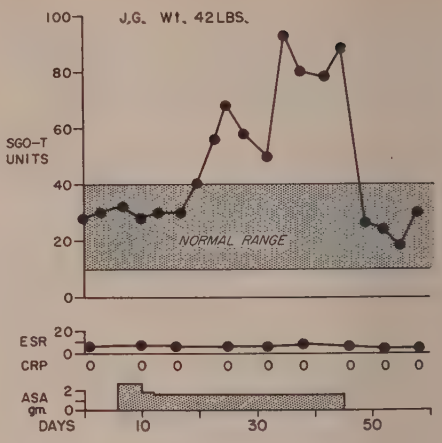

FIG. 1.

final concentrations of 5 to 40 mg %. The SGO-T and SGP-T activities of the sera were measured and compared with those of the same sera before the addition of these drugs.

Results. The effects of the administration of aspirin and sodium salicylate on serum transaminase activities in the patients of the 2 groups studied are summarized in Tables I and II. All patients had normal enzyme activities in their sera in the control period, before the administration of aspirin or of sodium salicylate. It is apparent that of 12 children in the first group receiving aspirin, 6 showed elevations of SGO-T to 50 units or greater. The maximal rise was to a level of 146 units. Three of these patients had no evidence of rheumatic activity and 3 were considered to have possible activity.

One of the 2 children receiving sodium salicylate showed an elevation of the SGO-T to 100 units. This patient did not have active rheumatic fever. The concentration of SGO-T in the other patient rose to 44 units, a borderline value.

A typical case is illustrated in Fig. 1. J.G., a patient convalescent from rheumatic fever and without evidence of rheumatic activity was maintained on aspirin, 1.2 to 2.4 g daily for more than one month. The SGO-transaminase first rose to an abnormal concentration 2 weeks after initiation of therapy, reaching a peak of 93 units 26 days after the first dose of aspirin. The SGO-T returned to a normal value within 2 days after discontinuation of the drug.

TABLE II. Effect of Aspirin Administration on Serum Glutamic Oxaloacetic and Serum Glutamic Pyruvic Transaminases.

Patient	Rheumatic activity	No. of days of aspirin admin. previous to 1st significant rise		Maximal level	
		SGO-T	SGP-T	SGO-T	SGP-T
1*	No	7	11	540	900
2	"	10	14	180	140
3	"	13	13	120	216
4	"	no rise	no rise	<50	<45
5	"	13	13	62	50
6	"	22	22	64	45
7	"	no rise	no rise	<50	<45
8	?	"	"	<50	<45
9	Yes	6	6	74	76

* This patient suffered from infectious hepatitis 18 mo earlier.

Table II shows the simultaneous variations of both SGO-T and SGP-T in the second group of 9 children in whom aspirin was administered until the appearance of toxic symptoms. Six of the children developed elevations of both SGO-T and SGP-T. Maximal levels of 540 SGO-T units and 900 SGP-T units were noted. These occurred in a patient who had had infectious hepatitis 18 months earlier. The shortest duration of aspirin administration resulting in significant rises of either enzyme was 6 days. Twenty-two days of continuous administration elapsed before the first elevation in patient No. 6,

whereas patient No. 7 received aspirin for 32 days with no detectable rise in the serum activity of either transaminase, despite comparable dosages and serum salicylate levels. A second transitory rise of one or both enzymes was noted in 4 cases 3 to 10 days after cessation of aspirin therapy at a time when there was no detectable salicylate in the patients' sera (Fig. 2).

No consistent relationship between serum salicylate levels and serum enzyme activities was found. There was no evident correlation between changes in the indices of inflammation (erythrocyte sedimentation rate, white blood cell count and C-reactive protein) and SGO-T or SGP-T.

No change in the rate of the transamination reaction of the test solutions or of the optical density of reduced coenzyme I was produced by the *in vitro* addition of aspirin or of sodium salicylate. The concentrations of salicylate tested encompassed the range of concentrations measured in the sera of the patients studied.

Discussion. It is clear that aspirin administration can influence the serum activity of glutamic oxaloacetic and glutamic pyruvic transaminases in children. For this reason any increase in the serum activity of these enzymes during the course of rheumatic fever should be evaluated with great care when aspirin is employed in the therapy of these patients.

The mechanisms whereby this aspirin effect is mediated are unknown at the present time. A direct effect of aspirin upon the rate of the transamination reaction in the test mixture or upon the optical density of reduced coenzyme I is ruled out by the *in vitro* studies mentioned. Among the several other possible explanations the possibility of an increased release of these enzymes from the liver as a result of aspirin administration deserves consideration. Experimental and clinical studies have documented changes in hepatic function and morphology following aspirin administration(12). SGO-T has been shown to be a sensitive and early indicator of hepatic damage(5). Furthermore, the simultaneous elevation of SGO-T and SGP-T is consistent

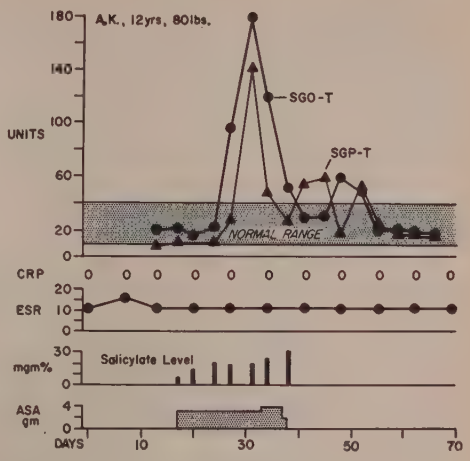

FIG. 2.

with the possibility that the liver is the source of the increase of the serum activity of these enzymes.

Summary. The effect of aspirin and of sodium salicylate administration on the serum glutamic oxaloacetic transaminase activity in 14 children and its effect on both the serum glutamic oxaloacetic transaminase and the serum glutamic pyruvic transaminase in an additional 9 children has been studied. Elevations to abnormal levels were observed in more than 50% of the cases. The importance of this finding in relation to the interpretation of the serum glutamic oxaloacetic transaminase elevations in rheumatic fever is pointed out and some possible underlying reasons for this phenomenon are mentioned.

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Corn Oil and Hypercholesteremic Response in the Cholesterol-Fed Chick.* (22672)

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Recently Ahrens(1,2), Kinsell(3,4,5,6) and Bronte-Stewart(7) and their coworkers have reported unusual reductions of the serum cholesterol in subjects receiving a formula diet in which various vegetable oils, in rather large quantities, were the main source of fat. Ahrens, *et al.*,(2) demonstrated that corn oil was the most effective of these agents in maintaining a low level of serum cholesterol. They also have shown a correlation between the iodine number and the hypocholesteremic effect of several vegetable oils, and have postulated that the degree of unsaturation of the fatty acids is responsible for that effect. Meanwhile, we had noted a lower level of serum cholesterol in cholesterol-fed control chicks used to assay fractions of a hypocholesteremic brain extract(8). This difference could be related to the fact that we had begun to use corn oil instead of cottonseed oil as a vehicle for the administration of cholesterol in the atherogenic diet.

The following experiments were performed in an effort to test the hypothesis that it is the slightly higher level of unsaturated fatty acids in corn oil which distinguishes it from cottonseed oil in its effect on the cholesterol-fed bird.

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Methods. In each experiment, 40 8-week-old White Rock cockerels were divided into 4 dietary groups, each of which were offered equal weights of the diets. Diet consumption was estimated daily, body weight weekly. Two birds losing weight and apparently ill were excluded from the data. All of the diet offered was eaten, except as noted below. The chicks were bled from the alar vein at 2-week intervals, and the plasma analyzed for serum cholesterol by the method of Abell, *et al.*(9). At termination of the experiments, the aortae were examined for gross atherosclerotic plaques and graded as described by Horlick and Katz(10).

Experiment 1: To test the importance of the relative proportions of oleic and linoleic acids as they occur in corn oil as opposed to cottonseed oil, we used 2 "neo-fat" fatty acid products of Armour Chemical Division. Neo-Fat 105 consists of fatty acids redistilled from the cottonseed oil; Neo-Fat 110 is also derived from cottonseed oil fatty acids but fractionated so as to approximate the same relative proportions of palmitic, oleic and linoleic acids as occur in corn oil. According to the producers, these 2 distillates had the same minor difference in iodine number seen between corn oil and cottonseed oil, but neither had any myristic acid, which is normally present in corn oil. Each contained approximately 2% unsaponifiable matter derived from cottonseed oil. One group was fed 10% solvent extracted corn oil (Mazola); the second group 10% cottonseed oil (Puritan Oil, Procter and Gam-

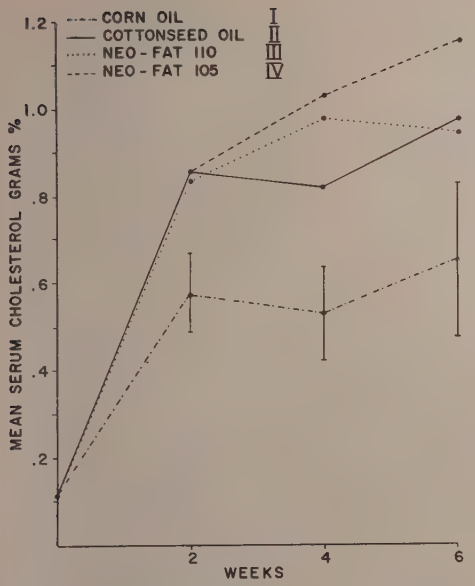

FIG. 1. Mean serum cholesterol in the 4 dietary groups indicated above. Vertical lines indicate ± one stand. dev. in the corn oil fed birds.

ble). The third group was fed 10% Neo-Fat #105, the fourth, 10% Neo-Fat #110 plus 0.1% myristic acid; each diet contained 1% cholesterol and Kay Bee chick starter mash. The latter contains ground corn, oats and wheat bran, plus alfalfa and soy bean meals which provide no less than 3% fat.

Experiment 2: Group I was fed 17.8% whole corn germ (containing 56% lipid material); Group II was fed 10% crude corn oil, expressed mechanically from the whole germ; Group III was fed solvent (hexane) extracted corn oil; and Group IV received 8% solvent extracted germ plus 9.8% cottonseed oil.‡ Thus each group received 10% lipid material by weight. One percent cholesterol was added to all diets, mixed in the oil or, in the case of whole germ, added in crystalline form. Stammler *et al.*(11) have shown that the mode of addition of cholesterol to the diet is not crucial. Each diet was mixed for at least 20 minutes in a Hobart mixer.

‡ We are grateful to Dr. Artz of Corn Products Refining Co., Chemical Division, Argo, Ill. who supplied us with these preparations and their specifications.

Results. The results of the first experiment may be seen in Fig. 1. The birds fed corn oil had a consistently lower serum cholesterol level than those fed cottonseed oil or either of its fatty acid distillates. This difference was statistically significant ($P<0.01$) at each of the 3 bleedings. No statistical significance could be found between mean plasma cholesterol levels at any bleeding of the birds fed cottonseed oil, as compared with its 2 distilled fatty acid products, though both of the distillation products tended to be more conducive of hypercholesteremia under these conditions than the original cottonseed oil. No significant difference in incidence of atherosclerosis was observed though the average grade of severity tended to correspond with the mean level of serum cholesterol.

The second experiment was devised to investigate a cruder preparation of corn oil and the whole corn germ. The cholesterol levels for the 4 groups are plotted in Fig. 2. The whole germ fed birds were significantly lower than all other groups at each bleeding, manifesting a very minimal elevation of the cholesterol level. Statistically, the level of probability is less than 0.01% in the last 2 bleed-

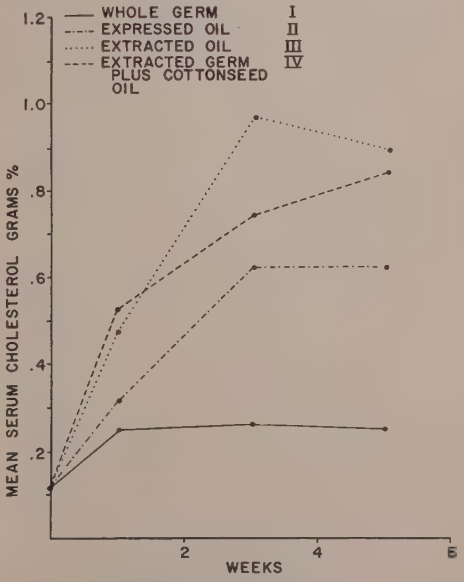

FIG. 2. Mean serum cholesterol in the 4 dietary groups indicated.

TABLE I. Estimated Proportions of Dietary Foodstuffs and Degree of Hypercholesteremia and Atherosclerosis Observed Terminally in the 4 Dietary Groups.

	Source of added fat	Grams/100 g of diet estimated Fat	CHO	Protein	Terminal mean plasma cholesterol ± S.E.	Terminal mean atherosclerosis, aorta-grade (+ to 4+)
I.	17.8% whole corn germ	12.4	42.0	18.0	251 ± 29.1	.2
II.	10% crude expressed corn oil	13.0	44.5	17.8	632 ± 98.5	.6
III.	10% solvent extracted corn oil	13.0	44.5	17.8	905 ± 127	1.0
IV.	8% solvent extracted corn germ; 9.8% cottonseed oil	13.4	45.3	18.2	850 ± 112	1.0

ings. The cholesterol level is significantly lower in the expressed oil fed group than in the extracted oil group at the one and the 3-week bleedings ($P<0.05$), but not at the terminal bleeding. In this experiment the birds in Group III ate only 96%, and in Group IV, 88% of the diet offered. Groups I and II ate the full 100 g allowed per bird per day. It is doubtful that the difference between Groups II and III could be explained by the difference in cholesterol intake, though this may well explain why Group IV did not develop still higher levels of plasma cholesterol. The serum cholesterol levels achieved here are higher with corn oil than in the first experiment because of a greater dietary allowance of 100 g/day of the respective diets as opposed to 75 g/day per bird.

The results of grading the aortae of all birds in this experiment are shown in Table I. It can be seen, as with the plasma cholesterol levels, that there is a difference of borderline significance between the oil-fed groups; but the group getting whole germ is significantly protected against the early lesions seen in the other groups.

Discussion. Ahrens and coworkers(2) were able to correlate the iodine number roughly with the serum cholesterol-lowering effect, but other possible factors were not investigated. Certainly the data of Bronte-Stewart and his coworkers(7) confirms and more strongly suggests the importance of saturation of the fatty acids for the hypercholesteremic response to cholesterol feeding in the human. Swell and Flick(12) compared the response of the serum cholesterol in rats to cholesterol feeding in conjunction with lard, oleic acid or stearic acid, and concluded that the absorption of cholesterol appears to be decreased with a highly saturated fat. Later Swell and coworkers(13) showed substantial differences in the degree of elevation of the serum cholesterol levels of rats fed equal amounts of various fat sources. Here they found the response to be greatest in the descending order: Oleic acid, stearic acid, linoleic acid, peanut oil, corn oil and linseed oil. They also demonstrated that *in vitro* esterification of cholesterol proceeded more rapidly with fatty acids than with neutral fat.

These experiments leave little doubt that the serum cholesterol level is greatly influenced by the nature of dietary fat employed. They also reach fairly general agreement that corn oil seems to promote cholesterol absorption less than many other fats or fatty acid combinations, though Lin and coworkers(14) suggested that tripalmitin or other poorly utilized fats might be used even more profitably to effect a lower serum cholesterol. Our first experiment demonstrates that corn oil does not permit as great a hypercholesteremia as cottonseed oil, and further that this difference cannot be explained by the slight differences in fatty acid composition between these two oils. While there is nothing conclusive about our second experiment, it too suggests that the degree of saturation of the fatty acids is not the sole factor in determining the response to cholesterol feeding. Virtually all of the neutral fat is obtained from the corn germ by these two methods of extraction and it is doubtful that the fatty acids occur in any different proportions in the residue than in the expressed oil. The waxes and the unsaponifiable fraction are more apt to be left behind. It is doubtful that sitosterols could explain the effect, for they occur as only approximately one percent of the oil, (or 0.1

g per chick per day).

The various oils and corn germ products were added to the mash so that the fat level was essentially equal in each group, and it can be seen from Table I that protein and carbohydrate also remained in about the same proportion. Portman, *et al.*(15) have shown that the quality of dietary carbohydrate can be important in determining the hypercholesteremic response in cholesterol-fed rats. They found that corn starch in the diet inhibited the rise usually seen with simpler sugars. Certainly this might be a partial explanation for the low levels of cholesterol seen in Group I, but Group IV received an equal supplement of corn starch in the extracted germ without any impairment of the hypercholesteremic response. It seems most likely that an unidentified fraction of the germ oil, not easily extracted, is inhibiting the expected rise in serum cholesterol.

Summary. Dietary experiments in the cholesterol-fed chick have demonstrated that a higher level of serum cholesterol is achieved when cottonseed oil is fed instead of corn oil. Reconstituting the distilled fatty acids of cottonseed oil, and adding a small amount of myristic acid so that the proportions of the major fatty acids in corn oil were observed, did not make cottonseed oil fatty acids behave like corn oil in restricting the rise in serum cholesterol. Comparison of the whole corn germ with expressed and solvent extracted corn oil revealed that it was a much more potent agent for limiting the hypercholesteremia and subsequent atherosclerosis than the more refined products. It seems unlikely that this difference between corn oil and cottonseed oil in the effect on the hypercholesteremic response of the cholesterol fed bird could be explained by a difference in fatty acid composition.

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Studies on Connective Tissue of the Cock Comb. II. Effect of Androgens.* (22673)

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Although the capon comb test has been used extensively for the biological assay of androgens, relatively little is known of the mechanism of this reaction in terms of tissue structure. The appearance of a so-called mucoid connective tissue has been recognized as the final result of hormone stimulation(1). An increase in the water content of the comb has been observed and is considered to be an important factor in the volume increase; based on this, the term "expansion growth" has been suggested for this reaction(2,3). Proliferation of the connective tissue cells does not seem to contribute significantly to the volume increase(3). Earlier histological studies indicated that mucopolysaccharides are present in the ground substance of the connective tissue of the rooster comb; this has been confirmed by the isolation of hyaluronic acid from the comb(4). It has been found that testosterone treatment increases the hexosamine content of the young chicken comb(5), but no similar studies have been reported on the capon comb after hormone treatment. Comparing the hexosamine content of the capon with that of the rooster comb, we found considerably higher values in the latter, while histochemical evidence indicated that acid mucopolysaccharides are present only in the rooster comb(6). The aim of the present study was to investigate the histochemical changes in the capon comb after testosterone treatment and to correlate these changes with the results obtained from determinations of the hexosamine, hydroxyproline and water content of the comb. The other head appendices, *i.e.* the wattles and the earlobes, have also been included in this study. Both are known to react to hormone treatment. However, while the comb and the wattles are similar in structure, the earlobes are made up of a morphologically different tissue. We also extended this investigation to the study of the normal development of the comb in untreated young cockerels.

Material and methods. 18 White Leghorn capons, approximately one year old were castrated at the age of 4 to 6 weeks and treated, with the exception of 4 control animals, with daily doses of 2 mg testosterone propionate (*"Neohombreol," Organon*)‡ for periods of 3 to 13 days. For comparison, the data obtained on 10 White Leghorn roosters of the same age are included. The development of the comb was studied in cockerels from the time they were hatched until 15 weeks of age, using a total of 82 animals.

Sampling the comb for a comparative study of its different stadia presents some difficulty due to the peculiar form of this organ and its anisometric volume increase. This is illustrated in Fig. 1, where the gradual increase in the area of the capon comb during hormone treatment is shown. It is obvious that in the course of this process each point of the comb moves at a different speed and in a different direction. By taking transverse slices from the comb at the plane of arrow A (Fig. 1), one can obtain sections which represent analogous parts of the comb in different animals. In studying such sections of the capon comb after different periods of hormone treatment, we found that the changes in the connective tissue start at the base of the comb and proceed upward from the skull toward the spikes(3). Such transverse sections might have obvious advantages for a morphological and a histochemical study. However, because the composition of these sections is not uniform, correlation of the results of chemical analysis

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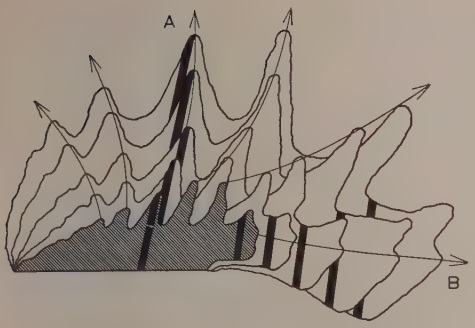

FIG. 1. Schematic representation of increase of comb area in the capon in the course of testosterone treatment (2 mg daily). Based on contact photographs taken every second day during first 8 days of treatment. Heavy black lines indicate location of samples.

with the histological structure is much more difficult. For this latter purpose, we used comb slices cut perpendicularly to arrow B (Fig. 1), which represent a more homogeneous cross section of the process at different time intervals.

The histological and histochemical methods, including the sectioning of the fresh frozen tissue, the periodic acid-Schiff reaction (PAS) and metachromatic staining with toluidine blue, have been described in detail(6). In a few cases the tissue, after fixation in Pfuhl's mixture (sublimate, picric acid, glacial acetic acid), was embedded in paraffin and stained with haematoxylin-phloxin or azocarmin-orange G-aniline blue (AZAN). Hexosamine determinations were carried out according to the modified Elson-Morgan method described previously(6). Comb slices of about 300 mg were used for this purpose except in very young cockerels, where it was necessary to use the whole comb or even pooled samples of several combs. Prior to hydrolysis, the tissue samples were extracted by acetone and dried in a desiccator to constant weight. This dry weight of the defatted tissue has been used to calculate the water content. The method described by Neuman and Logan(7) has been used for the determination of the hydroxyproline content. The data reported on the hexosamine, hydroxyproline and water content represent averages of determinations made on 10 combs of roosters, 4 combs of untreated capons, and 2 combs from each group of treated capons; while in the case of cockerels, the number of combs used varied between 3 and 8. The ratio of hexosamine to hydroxyproline has also been calculated.

Results. Testosterone-treated capons. The average weight of the untreated capon comb was 1.57 g, while the rooster combs measured about 45 g. After 13 days of testosterone treatment of capons, the average comb weight was 21.2 g. A detailed description of the connective tissue of untreated capon comb is given in previous reports(3,6); here we shall mention only that there is no metachromatic ground substance present in the capon comb and that the hexosamine content is rather low (0.53% dry weight). Estimating the hydroxyproline content in the comb of untreated capons, we found an average value of 3.65% dry weight, resulting in a ratio hexosamine: hydroxyproline of 0.15. Studying the changes in the capon comb after testosterone treatment, we found after 3 days that there was already a large amount of metachromatic material present in the intermediate layer, especially in the lower parts of the comb. The gradual development of this mucoid tissue is shown in Fig. 2, 3 and 4, which are taken from different parts of the same transverse section through the middle of the comb. Fig. 2 shows the very first appearance of the metachromasia in the spike; Fig. 3 shows further development in the middle part; while Fig. 4 represents a fully developed mucoid tissue at the base of the comb close to the skull. In comparing sections from the combs of capons after different periods of treatment, 3, 5 and 13 days respectively, we found that after 3 days the fully developed mucoid tissue is restricted to the basal parts, while after 5 days almost the whole section contains this layer. Finally, after 13 days, the intermediate layer throughout the entire comb is transformed into mucoid tissue, occupying all of the space between the reticular layer under the epithelium and the central core. The appearance of the metachromasia is the main occurrence histochemically. We did not find any change in the PAS positivity, which was restricted to the collagen fibers(6).

The wattles show essentially the same

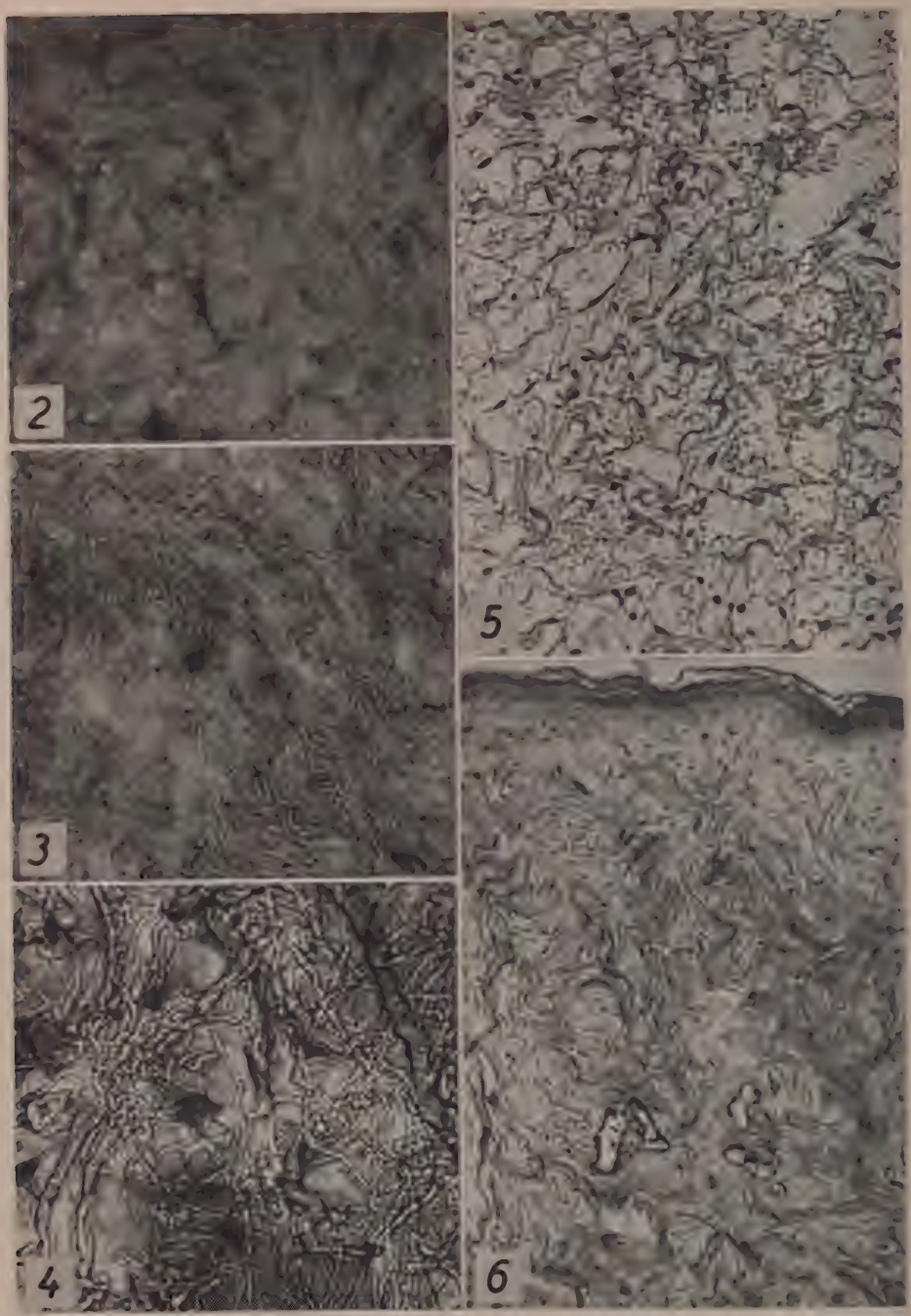

FIG. 2. Capon comb after 3 days of hormone treatment. Frozen section 12.5 μ, fixation in osmic vapor and lead subacetate, stained with toluidine blue (magnification 360×). Mucoid layer in the spike.

FIG. 3. Same section and magnification as in Fig. 2. Mucoid layer in middle part of comb.

FIG. 4. Same section and magnification as in Fig. 2. Mucoid layer in basal part of comb near skull.

FIG. 5. Subcutaneous connective tissue of the wattle of a capon after 3 days of testosterone treatment, showing edema. Fixation: Pfuhl, paraffin section 8 μ, stained with AZAN (magnification 320×).

FIG. 6. Earlobe of untreated one-year-old rooster. Fixation: Pfuhl, paraffin section 6 μ, stained with haematoxylin-phloxin (magnification 180×). Note absence of blood vessels under epithelium and presence of thick, collagen bundles in dermal layer.

structure as the comb, both in the capon and in the rooster, and the reaction to testosterone treatment is very similar. It should be mentioned that the mucoid layer here is narrower than in the comb. Therefore, the mucoid tissue represents a smaller portion of the total structure. There is no preferential localization of the very first metachromatic change in the wattles, as in the basal part of the comb. We noticed another way in which the reaction here seems to differ from that in the comb; *viz.* the edematous appearance of the subcutaneous tissue of the wattle, which was especially pronounced during the first days of hormone treatment. At this time, the wattle increases markedly in thickness, and on cutting through the tissue, fluid escapes from the central part. This fluid showed strong metachromasia when toluidine blue was added *in vitro,* but it was technically impossible to demonstrate this substance in the tissue sections. The spaces between the fibrils of this edematous middle part appear empty in the histological preparation (Fig. 5). Later on this edema tends to disappear; after 13 days of treatment, the subcutaneous tissue of the wattles appears to be similar to the structure in the rooster without any sign of edema.

Histologically, the earlobes of both the capon and the rooster differ from the comb and wattle in many ways. In the subepithelial part of the rooster comb and wattle, the so-called reticular layer, there is a large number of sinusoid blood vessels, showing dilatation upon hormone treatment and giving these organs their characteristic red color. These subepithelial vessels are absent in the earlobes, where there is only a small strip of tissue somewhat similar to the reticular layer of the comb and wattle. While the dermal layer of the comb and wattle of the normal rooster and treated capon contains thin collagen fibrils, the dermal layer of the earlobes consists of thick collagen bundles, homogeneous in appearance. A few small, coiled blood vessels are also present here. (Fig. 6). Compared to the very profound reversible changes of the dermal layer of the comb under hormone treatment, the dermal layer of the earlobes shows fewer alterations. In the capon, the whole dermal layer is narrower and there is no metachromasia present. In the testosterone-treated capon and in the normal rooster, this layer is wider, the collagen bundles have a swollen appearance, and diffuse metachromasia, very faint but still distinct, is present throughout the whole dermis. The only effect of hormone treatment on the earlobe which is similar to that on the wattle is some accumulation of fluid in the subcutaneous tissue, but to a much lesser extent.

The results of chemical analysis of all 3 tissues in the treated capons are summarized in Fig. 7. It is obvious that there is a significant increase in the total hexosamine content of the capon comb during hormone treatment. The same is true for the wattles, but to a lesser degree, while in the earlobes the increase in hexosamine, if at all significant, is very slight. The relative amount of hydroxyproline does not change significantly in the capon comb during hormone treatment; this also seems to apply to the wattles and earlobes. When these values are related to each other, as the hexosamine:hydroxyproline ratio, it is obvious that this ratio tends to increase in all 3 organs during testosterone treatment; these values can be even higher than in the normal rooster.

Normal development of the comb in cockerels. In our experiments, the comb weight increased from 0.013 g at the time of hatching to about 15 g at the age of 15 weeks. The histological structure of the comb in a one-day-old cockerel shows a cellular proliferation of the connective tissue of the dermal layer;

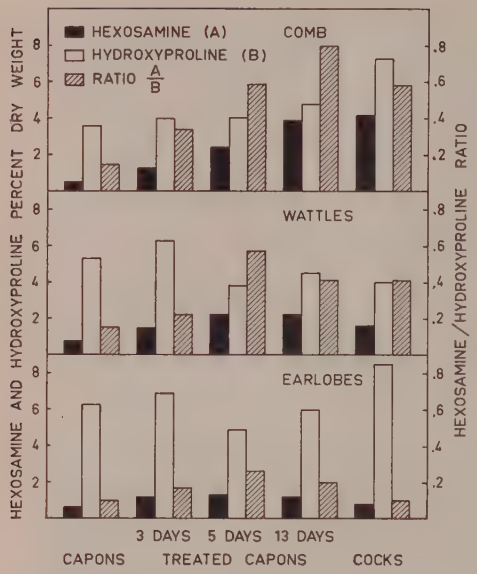

FIG. 7. Hexosamine and hydroxyproline content and hexosamine-hydroxyproline ratio in comb, wattles and earlobes of testosterone-treated capons. For comparison, values from untreated capons and roosters of the same age are included.

the collagen fibrils are already developed, but there is no metachromasia in the ground substance. This picture does not change essentially during the first 3-4 weeks, which seems to cover the normal growth period judging from the frequent mitoses observed both in epithelium and in connective tissue. After this period the accumulation of the metachromatic ground substance begins and is again more pronounced in the basal parts of the comb. Until the age of 15 weeks there is a gradual increase in the amount of mucoid tissue in the comb. During this time mitoses still can be seen in the epithelium, but are seldom observed in the connective tissue. At the end of this period the structure of the normal rooster comb has been developed. Chemical determinations (Fig. 8) show that the relative amount of hexosamine in the comb starts to increase very slowly during the first 4 weeks, but increases more rapidly thereafter, reaching the level of the fullgrown rooster comb in about 15 weeks. The relative amount of hydroxyproline is constant throughout the whole development. The hexosamine-hydroxyproline ratio conceivably

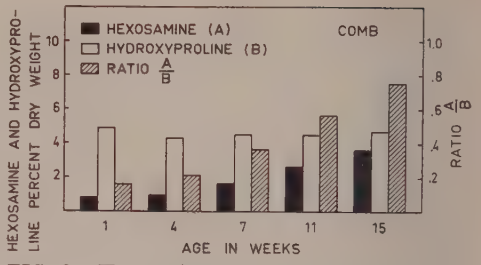

FIG. 8. Hexosamine and hydroxyproline content and hexosamine-hydroxyproline ratio in comb of developing cockerels.

increases during this time.

In Fig. 9 water content data of comb samples are plotted against the corresponding hexosamine values. These figures show that in treated capons the water content is somewhat higher than in normal, untreated roosters and older cockerels.

Discussion. The comparative histological study of the comb development in cockerels and in testosterone-treated capons showed that in both cases the process of "expansion growth" is essentially similar. This process takes 10 to 15 weeks in cockerels; in treated capons it can be completed in about 2 weeks.

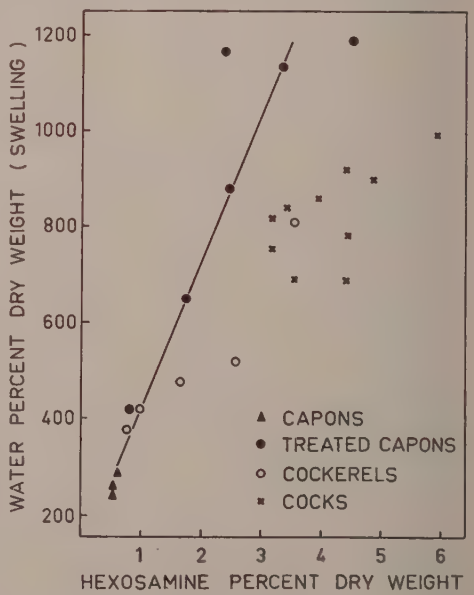

FIG. 9. Water content (expressed in % of fat-free dry wt) and hexosamine content of comb in treated and untreated animals.

In the cockerel, it is preceded by a phase of normal tissue growth which takes place during the first 3-4 weeks after hatching. Mitoses in the connective tissue were found only during the first 4 weeks; after that, during the "expansion growth" period, we rarely found cell division in the mucoid layer. Ludwig and Boas(8) and Schiller, Benditt and Dorfman (5) observed active cell proliferation in the connective tissue of the comb in young cockerels receiving androgen treatment. The animals used were 3-24 days old(8) and 10 days old(5) respectively. A possible explanation for this difference could be that in animals so young the process of "expansion growth" overlaps the normal growth process. Determination of the total hexosamine shows that there is an increase of some hexosamine-containing substance or substances in both treated capons and normally developing cockerels in the course of the "expansion growth." This applies especially to the comb, but also seems to occur in the wattles and possibly in the earlobes. It should be mentioned that our data do not allow any further interpretation of this increase in hexosamine content, but they are in all cases parallel to the metachromasia as found under the conditions of the histochemical test. This altogether might be evaluated as indirect evidence for the supposition that one of the main phenomena in the hormonal response of these tissues is the accumulation of mucopolysaccharides in the ground substance, probably hyaluronic acid. Since the concentration of hydroxyproline in the treated capon remains relatively constant in the course of the "expansion growth," this indicates that there is also an active formation of collagen during the process which is proportional to the increase of the total fat-free dry mass of the comb. Compared to this the increase in hexosamine content is much larger. The same applies to the increase in water content, which is parallel to the increase in hexosamine content, suggesting that these two phenomena are in some way related to each other(2). This is supported indirectly by the previous observation that the water content of the isolated mucoid layer of the rooster comb as well as its hexosamine content are much higher than the values obtained on the total comb(6). Another factor which might partially explain the high water content of these organs is the subcutaneous edema which develops in the course of hormone treatment, especially in the wattles and earlobes. Finally, the vasodilatation will increase the blood content of the tissue and also may contribute to the high water content.

Summary. It has been found that the increase of the metachromasia in the connective tissue of the comb coincides with the increase of the total hexosamine content during the development of this organ in the normal rooster, as well as during the testosterone treatment of the capon. In both cases, there is an increase in the water content, suggesting that the presence of mucopolysaccharides in this tissue and the high water content are related phenomena. The relative hydroxyproline values remain essentially unchanged during this reaction, indicating that increase in collagen content is proportional to total increase of the dry substance. The ratio hexosamine:hydroxyproline thus shows an increase in both cases. A similar increase was also found in the wattles and, to a lesser degree, in the earlobes. The wattles show the same structure and reaction as the comb. In the earlobes there is a very slight increase of the metachromasia in the course of hormone treatment which, together with the slight increase in the hexosamine:hydroxyproline ratio, would suggest that the reaction here is also similar, but much less pronounced. During testosterone treatment a transient edema of the subcutaneous tissue has been found, especially in the wattles and earlobes.

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Effect of X-Ray Dose on Mortality and Skin Transplantability in Mice Receiving F_1 Hybrid Marrow.* (22674)

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Mice receiving an otherwise lethal dose of X-irradiation, but protected with bone marrow from an F_1 hybrid derived from the irradiated and a foreign strain, will subsequently tolerate skin homografts from the F_1 hybrid or the foreign parent strain of the F_1 hybrid(1,2). Mice protected with marrow from the same (isologous) strain will not tolerate skin homografts from the F_1 hybrid or the foreign parent strain. The following experiments were undertaken to determine the lowest dose of whole body X-irradiation, followed by marrow from an F_1 hybrid, that would result in tolerance of skin homografts from the foreign parent strain. During the course of these experiments an entirely unexpected and interesting observation was made regarding mortality in marrow-treated mice at decreasing doses of irradiation.

Materials, methods and terminology are similar to those reported previously(2) with the following exceptions. One marrow donor

TABLE I. Percent Mortality following X-irradiation.

	All strains combined			CBA strain		
		% mortality after indicated			% mortality after indicated	
Dose, r	No. of mice	No. of days 21	30	No. of mice	No. of days 21	30
330	22	0	0	12	0	0
400	12	0	0			
500	24	17	21			
550	54	9	9	24	12	12
600	36	64	67	12	50	50
660	84	93	94	48	90	90
700	24	100	100			
770	266*	99	100	60	100	100
800	50	100	100	11	100	100
1000	12	100	100			
1200	12	100	100			

* 159 of the mice at 770 r received intrav. saline inj. as controls for other groups receiving bone marrow suspensions.

TABLE II. Mortality and Skin Transplantability in Unirradiated, X-irradiated and Marrow Treated CBA Mice.

				% mortality after indicated No. of days					Skin graft				
Exp.	X-ray dose, r	Marrow donor	No. of mice	12	21	30	60	100	Skin donor strain	No. of mice grafted	Days post-X-ray and/or marrow when grafted	No. of takes	No. of sloughs
A	0	Cb × CBA	12	0	0	0	0		Cb	12	46–52	0	12
	110	*Idem*	12	0	0	0	0		"	12	46–52	0	12
	330	"	12	0	0	0	0		"	12	46–52	0	12
	550	"	12	67	100	100	100	100		0			
	770	"	12	0	0	0	8*		Cb	11*	46–52	11	0
	0	None							CBA	13		12	1
B	660	Cb × CBA	12	0	0	17	42†	50	Cb	9	34–55	6†	0
	770	*Idem*	12	0	0	0	0	0	"	12	34–55	9	3
	660	None	12	67	100	100	100	100		0			
	0	"							Cb	9		0	9
	0	"							CBA	21		21	0

* One mouse in this group died the day following skin grafting and is excluded from the skin graft data but included in the mortality data.

† Three mice in this group died (one as a result of anesthesia during photography) with viable skin grafts 13 to 18 days after skin grafting. They are excluded from the skin graft data but included in the mortality data.

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† The author is indebted to Dr. R. Shalek for assistance in dosimetry, and to M. A. Flores and B. Morris for technical assistance. X-ray facilities were generously provided by the M. D. Anderson Hospital and Tumor Institute.

TABLE III. Mortality and Blood Count in X-irradiated CBA Mice Receiving Cb × CBA F_1 Marrow.

Strain of mice	X-ray dose, r	Marrow donor	No. of mice	% mortality after indicated No. of days			Blood counts at 2 wk post-irradiation					
				12	21	30	No. of mice counted	WBC/mm³ Mean	WBC/mm³ Low	WBC/mm³ High	Million RBC/mm³ Low	Million RBC/mm³ High
CBA	330	Cb × CBA	12	0	0	0						
"	550	*Idem*	12	33	92	92	5	3,430	800	7,400	3.18	11.75
"	770	"	12	8	8	17	5	9,190	8,500	11,950	8.65	13.63
Balb/c*	770	None	12	100	100	100						

* Balb/c mice were used as an irradiation control because of a shortage of CBA mice.

FIG. 1. Body wt data of Exp. A, Table II. All points represent mean wt of 12 mice with the exception of the 550 r group. Numbers along this line represent decreasing numbers of surviving mice at each point.

mouse was used for every 2 irradiated mice in Experiment A of Table II and in the experiment of Table III, rather than 1 for 1 as in previous work, including Experiment B of Table II. The intravenous injection volume was .5 cc throughout. The nucleated cell counts for the marrow suspensions used were 67,200, 89,150 and 61,250 per cu mm for Experiments A and B of Table II and for Table III respectively. Duco cement was applied to the sutured edges of the skin grafts of Experiment A, but not of Experiment B.

Observations. The 21-day LD_{50} of X-irradiation under the conditions used is about 600 r, while 770 r is uniformly lethal to all mice within 30 days (Table I) Mice of the CBA strain were equally distributed by age and sex into 5 treatment groups, each containing 12 mice. These groups received 0, 110, 330, 550, and 770 r of X-irradiation followed in 1 to 2 hours by intravenous injection of marrow from the Cb x CBA F_1 hybrid. As in past experience(2), F_1 marrow gave excellent protection against the otherwise lethal dose of 770 r (Experiment A of Table II). At the lower dose of 550 r, however, administration of the same suspension of hybrid marrow was followed by 100% mortality in 15 days. Body weight loss at 550 r was much more severe than at 770 r and was progressive until death (Fig. 1). Mice receiving still lower doses of irradiation or no irradiation, followed by marrow treatment, showed no mortality. At 46 to 52 days post-irradiation the surviving mice were homografted

with skin from the Cb strain, the other parent strain of the F_1 marrow donor. Of a group of 13 untreated CBA mice simultaneously autografted as controls, 12 of the autografts took. All of the homografts sloughed at the 0, 110 and 330 r dose levels. Of the 11 mice at 770 r that survived skin grafting, all of the foreign skin grafts were accepted and are in good condition 124 to 130 days after grafting. In previous experiments no late sloughs have been observed under similar conditions. Fifteen of 18 grafts at 660 and 770 r were successful and are still in good condition 130 to 152 days after grafting (Experiment B of Table II). In this earlier experiment it will be noted that late mortality in F_1 marrow protected mice at 660 r was greater than at 770 r. In both of these experiments mice receiving the different doses of irradiation were caged separately, 6 to a pen. It is therefore possible that an undetected infectious agent may have involved the 4 pens of mice at 550 and 660 r to account for the greater mortality at these levels than at 770 r. To control this possibility another experiment was set up in which mice receiving 330, 550 and 770 r followed by Cb x CBA marrow were caged together, 2 at each dose level in each pen of 6 mice. Again the mice at 550 r showed much higher mortality than their cage mates at 330 and 770 r (Table III). Body weight loss at 550 r was much greater and more progressive than at 770 r. Blood counts performed 2 weeks post-irradiation showed a significantly lower white and red cell count in the mice at 550 r than in those at 770 r. Platelets were less numerous in the stained blood smears of the mice at 550 r than in those at 770 r.

Discussion. The blood count data suggest that the greater mortality at 550 r than at 770 r may be related to a less adequate hematopoietic recovery. On the other hand, 550 r without bone marrow had earlier resulted in 30-day mortality of only 12% of 24 CBA mice and 9% of 54 mice of all strains combined. Additional experiments are obviously indicated, and no explanation of the mechanism involved will be ventured at this time.

Summary. The 21-day LD_{50} under the conditions of X-irradiation used is approximately 600 r. Irradiation of CBA mice followed by intravenous administration of bone marrow suspension from Cb x CBA F_1 hybrids resulted in little or no 21-day mortality at 110, 330, 660 and 770 r, but in almost 100% mortality at 550 r. Homografts of Cb skin into the surviving mice were accepted by the mice at 660 and 770 r dose levels but not by mice at 0, 110 and 330 r dose levels. None of the marrow-treated mice at 550 r survived long enough to be skin grafted.

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Effect of Chlorpromazine on Excretion of 5-Hydroxyindoleacetic Acid in a Patient with Malignant Carcinoid. (22675)

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Since the first authentic case report of Beger(1) in 1882 of a carcinoid of the appendix the tumor has been of interest to the clinician principally because of its rarity and to the histologist because of its unusual cell type and staining properties. Gosset and Masson(2), using silver impregnation technics, demonstrated the presence of intracytoplasmic granules which stain black with silver and which were indistinguishable from the granules found in the argentaffin cells of the intestinal mucosa. Masson suggested the possibility that carcinoid tumors arise from the argentaffin cells and postulated that the

normal cells had a neuroendocrine function.

Lembeck(3) demonstrated the presence of 5-hydroxytryptamine (5-HT) in carcinoid tumors in large quantities. This observation has led Thorson(4) to suggest that the secretion of 5-hydroxytryptamine (serotonin) by the tumor might serve to explain the unusual symptom complex seen in patients harboring this tumor. The clinical syndrome is characterized by intestinal hypermotility, bronchospasm, vasomotor disturbances and valvular disease of the heart. The recent report of Sjoerdsma(5) *et al.* concerning their investigation on a group of patients with malignant carcinoids support the postulate that the symptom complex described by Thorson and others is due to excess serotonin produced by the tumor. Further studies by the group have shown that 5-hydroxyindoleacetic acid (5-HIAA) is formed by oxidative deamination of serotonin and excreted in the urine(6). Increased amounts of this metabolite have been demonstrated in the urine of patients with malignant carcinoid and a method for its assay has been described by Udenfriend(7). The recent reports of Benditt(8) showing antagonism of 5-HT by chlorpromazine led us to study the effects of this drug on a patient with a known malignant carcinoid.

Method. The patient was a 56-year-old white woman in whom a carcinoid tumor of the ileum was diagnosed and resected in 1950. At the time of laparotomy metastases of the tumor to regional lymph nodes and liver were observed grossly and confirmed microscopically. She has enjoyed reasonably good health since that time with the exception of chronic diarrhea, occasional episodes of "flushing" and periodic exacerbations of "arthritis." No cardiac abnormalities have been noted. The patient was admitted to the Metabolic Division of University Hospitals and placed on a constant diet which remained unchanged during the study period. All urine was collected and aliquots from each 24-hour period were placed in a deep freeze. The number and character of the bowel movements were recorded for each 24 hours. Following a control period of 5½ days the patient was started on oral chlorpromazine,* 25 mg every 6 hours. This was continued for 4 days at which time the drug was discontinued. The observation period extended for an additional 4½ days following withdrawal of the medication. The 24-hour urine samples were assayed for 5-HIAA according to the simplified method of Sjoerdsma *et al.*(9). A gross quantitation of the amount of 5-HIAA excreted was made by the intensity of the color produced by the test and was adequate for our purpose. The patient received no other medication during the experimental period.

Results. Following administration of chlorpromazine there was a prompt and marked decrease in the excretion of 5-HIAA in the urine and a decrease in the number of bowel movements per 24 hours (Fig. 1). The stools became semi-solid in contrast to watery bowel movements observed during the control period. Within 24 hours following discontinuation of the medication the urine 5-HIAA content returned to control levels and the diarrhea again became manifest after 72 hours.

Discussion. The evidence that carcinoid tumors produce excess quantities of serotonin seems incontrovertible and patients with this lesion afford an unusual opportunity to study the pharmacodynamic effects of this substance *in vivo*. The patient described in this study may be added to a growing list of reported cases in whom the presence of this tumor is associated with unusual clinical manifestations which may be attributable to serotonin excess, *i.e.*, diarrhea, vasomotor disturbances (flushing), etc. The antagonistic effect of chlorpromazine on serotonin previously demonstrated in experimental animals(8) is further supported by this study as shown by the decrease in urinary 5-HIAA following administration of the drug. The decrease in intestinal hypermotility as manifested by the reduction in the number of stools noted in this patient affords additional corroborative evidence of serotonin inhibition. The mode of action of serotonin inhibition by chlorpromazine remains speculative. It is not to be inferred from this report that chlorpromazine is necessarily recommended in the clinical management of these patients albeit true that symptomatic relief of the distressing diarrhea

* Thorazine—Smith, Kline and French.

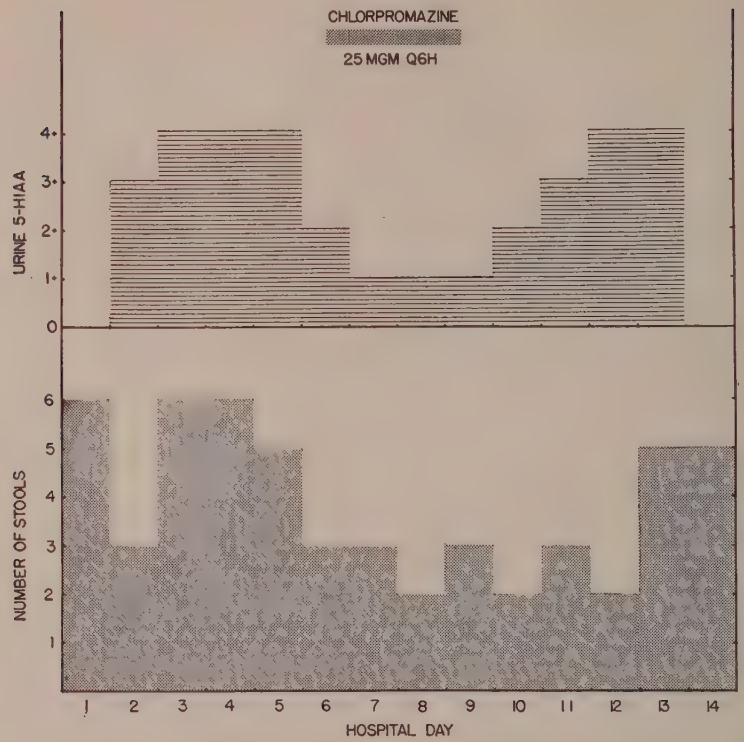

FIG. 1. Graph showing prompt fall in the urine 5-hydroxyindoleacetic acid following oral administration of chlorpromazine and a return to premedication levels following withdrawal of the medication. Concomitant diminution in the No. of stools is also depicted occurring with chlorpromazine medication.

was observed.

Summary. 1. A marked fall in urinary 5-HIAA excretion followed administration of chlorpromazine to a patient with known malignant carcinoid. 2. Intestinal hypermotility, one manifestation of the so-called "carcinoid syndrome" was likewise suppressed during administration of the drug.

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Stability of a Fluid Cholera Mucinase Preparation When Combined with a Commercially Prepared Cholera Vaccine. (22676)

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The demonstration by Burnet and Stone (1) that the mucinase of *Vibrio comma* causes desquamation of guinea pig intestinal epithelium suggested to these investigators that this enzyme may be involved in the pathogenesis of Asiatic cholera. Evidence to support this hypothesis is provided by the recent report of Lam, Mandle and Goodner(2) that the cholera mucinase increases the permeability of the mouse intestinal wall *in vivo* as well as *in vitro*. It is postulated that the mucinase, by causing desquamation of the intestinal epithelium, alters the permeability of the bowel wall to permit absorption of toxic substances and loss of fluids and electrolytes.

The usual cholera vaccine, which consists of non-viable vibrio cells, does not stimulate production of antimucinase when injected into animals(1,3). Vibrio culture filtrates containing active mucinase, on the other hand, are antigenic when administered parenterally to rabbits and guinea pigs, and result in the appearance of specific antibodies which inhibit the *in vitro* desquamation and depolymerization activities of the homologous mucinolytic enzyme(1,4). It would appear, then, that the addition of cholera mucinase to the vaccine would enhance the protective potency of this immunizing agent against an enteric cholera infection, provided the mucinase retains its antigenic properties in such a preparation.

Jensen(3) studied the stability of the cholera mucinase in culture filtrates and reported that the enzyme in this state is extremely sensitive to heat, storage and chemical preservatives. He demonstrated that *in vitro* enzyme activity and antigenicity are related, and that both properties can be preserved by lyophilization.

The studies reported herein were initiated to ascertain whether the application of a concentration and purification procedure would result in a stabilized fluid mucinase preparation, permitting the combination of the mucinase with the present cholera vaccine without affecting the *in vitro* activity and antigenicity of the enzyme, or the antigenicity of the somatic antigens.

Materials and methods. Culture: *V. comma* strain 20A78, the WRAIR designation for strain P93A obtained from Prof. K. Goodner, Jefferson Medical College, Philadelphia, Pa., was used for the preparation of mucinase by the method described below. *Cholera vaccine*. Lederle Cholera Vaccine, Lot #1976-67K (expiration date 21 Oct. 1955), was obtained in Nov. 1954 for use in these studies. The vaccine contained 8,000 million killed cholera vibrios (equal numbers of Inaba and Ogawa strains) per ml, preserved in 0.45% phenol. *Mucinase titrations*: *In vitro* mucinolytic activity was measured by the technic described elsewhere(5), which is based on the ability of the mucinase to depolymerize ovomucin so that the latter is no longer clotted by cetyl trimethyl ammonium bromide (CTAB). The ovomucin was prepared by the method of Gottschalk and Lind(6) and was standardized by the addition of borate-buffered calcium saline, pH 7.0,* to the maximum dilution containing sufficient dissolved ovomucin to form a characteristic fibrous clot on addition of one drop of 1% aqueous CTAB to 0.5 ml of the standard solution in 0.5 ml of buffered saline. The mucinase titers recorded are the reciprocals of the highest dilutions of the enzyme solutions which prevented the formation of the ovomucin-CTAB clot. *Antimucinase titrations*: The antimucinase activity of serum was measured by the constant antigen-varying antibody technic previously described (5). The titers recorded are the reciprocals of the highest serum dilutions which neutral-

* Borate-buffered calcium saline, pH 7.0: Per liter: 0.052 g $Na_2B_4O_7 \cdot 10H_2O$, 1.0 g $CaCl_2$ (anhydrous), 8.5 g NaCl, 1.203 g H_3BO_3.

ized the depolymerizing activity of 2 mucinase units, permitting the formation of the ovomucin-CTAB clot. *Agglutinin titrations*: Serum agglutinin titers were obtained by employing as antigen a saline suspension of agar-grown viable vibrios of the same strain from which the mucinase was prepared (20A78). Antigen-antiserum mixtures were incubated at 37°C for 2 hours followed by refrigeration overnight. The titers recorded are the reciprocals of the highest dilutions of the sera which yielded definite macroscopic agglutination.

Experimental results. Purification of Mucinase: In order to facilitate purification of the mucinase from culture filtrates an attempt was made to cultivate the cholera vibrio in a protein-free medium. Several synthetic media and media containing protein hydrolysates were tried, and although good yields of bacterial cells were obtained in many instances, little or no mucinolytic activity was demonstrated in the culture supernates. On the other hand, a dialysate of Brain-Heart Infusion Broth was found to support the growth of the organism and to yield satisfactory mucinase titers, and was consequently used as the culture medium.

Double strength Brain-Heart Infusion Broth (Difco) was dialyzed against an equal volume of distilled water at 4°C. After 24 hours the dialysate was collected and was replaced with another volume of water. This process was repeated again, after which the 3 resulting dialysate solutions were combined to constitute the medium for the growth of the vibrios. Nine liter bottles containing 2 liters of dialysate medium were seeded with 50 ml of a 6 hour vibrio culture (in the same medium) and were incubated at 37°C for 17 hours on a roller apparatus which constantly rotated the bottles (tilted at an angle of 30 degrees) at 40 revolutions per minute throughout the incubation period, providing maximum aeration without excessive foaming.

The bulk of the bacterial cells was removed by centrifugation and the remainder by filtration through a large Berkefeld filter of N (normal) porosity. The filtrate was then half-saturated with solid $(NH_4)_2SO_4$ and was placed in the cold room overnight. The resulting sediment was dissolved in borate-buffered calcium saline and was dialyzed against the buffered saline until the dialysate no longer precipitated with a saturated barium chloride solution. Sterilization of the product was accomplished by filtration through a Selas filter (02 porosity).

The final solution contained mucinase, other non-dialyzable ammonium sulfate precipitated extracellular products of vibrio metabolism, and cellular substances (somatic antigens, etc.) liberated through lysis of the cells. The material used in this study was prepared in May 1953 from culture filtrates of *V. comma* strain 20A78. The original culture filtrate contained 1.48 mg of nitrogen per ml and had a mucinolytic activity of 64 units per ml, while the final product contained 0.14 mg of nitrogen and 2048 mucinase units per ml (Table I). Comparable results have since been obtained with other preparations from the same strain, as well as with preparations from 4 other *V. comma* strains, including a recent isolate from a human cholera case.

Stability of in vitro enzyme activity: The partially purified mucinase in borate buffered calcium saline, prepared as described above, proved to be extremely stable when stored in the fluid state at 4°C, retaining its full *in vitro* enzymatic activity for at least 2½ years (column I, Table II). In Nov. 1954, after 1½ years storage, this mucinase solution was mixed with stock commercially prepared cholera vaccine in 4 different proportions. These mixtures, in rubber stoppered vaccine bottles, were stored at 4°C for an additional year, with periodic assays for *in vitro* mucinase activity. No significant variation in titer, be-

TABLE I. Comparison of Vibrio Culture Filtrate and Partially Purified Mucinase Solution.

Preparation	Vol, ml	Mucinase titer/ml	Mg N/ml	Mucinase units/mg N	Yield
Culture filtrate	7800	64	1.48	43.2	—
Purified mucinase	110	2048	.14	14629	45%

TABLE II. Mucinase Titers of Mucinase-Vaccine Mixtures.

	Mixture* No.				
	1	2	3	4	5
Mucinase solut'n†	20 ml	15 ml	10 ml	5 ml	1 ml
Cholera vaccine‡	0	5 "	10 "	15 "	19 "
Date tested	Mucinase titers				
5/18/53	2048	—	—	—	—
12/ 1/54	2048	1024	512	256	128
1/ 3/55	2048	1024	512	512	128
2/ 7/55	2048	2048	1024	512	128
3/21/55	2048	1024	1024	512	64
5/27/55	4096	2048	2048	1024	256
7/ 1/55	2048	1024	512	256	64
8/ 1/55	2048	1024	1024	512	64
9/30/55	2048	1024	1024	512	128
12/ 5/55	4096	2048	2048	1024	128

* Mixtures 2 through 5 prepared 11/24/54.
† Mucinase solution prepared in May 1953 from culture filtrate of *V. comma* strain 20A78.
‡ Lederle Cholera Vaccine, Lot No. 1976-67K. Mucinase titer = <4.

yond that inherent in a technic involving 2-fold serial dilutions of the enzyme solution and different preparations of the substrate, was noted with any of the mixtures during this period (Table II). *Stability of antigenic properties of the mucinase and of the somatic antigens*: Subsequent to storage at 4°C for one year, 3.25 ml of the mixture containing mucinase and vaccine in equal volumes was injected intravenously into 3 rabbits in 8 graded doses (ranging from 0.05 ml to 2.0 ml) at 3-4 day intervals. Simultaneously a second group of 3 rabbits received intravenous injections of 1.625 ml of the mucinase preparation alone and a third group was given 1.625 ml of the cholera vaccine alone, in graded doses ranging from 0.025 ml to 1.0 ml. The rabbits were bled 10 days following the final injection and the sera were assayed for agglutinin and antimucinase activity.

The antimucinase titers of the sera of rabbits immunized with the mucinase-vaccine mixture fell in the same range as those resulting from immunization with the mucinase solution alone (Table III). Thus there appears to be no decrease in the antigenic stability of mucinase as a result of its mixture with cholera vaccine, even after such a mixture has been stored for a year at 4°C. On the other hand, the appearance of agglutinins in the sera of these rabbits provides evidence that the presence of active mucinase in the vaccine does not destroy the antibody-stimulating properties of the vibrio somatic antigens.

The presence of agglutinins in the sera of the rabbits immunized with the mucinase solution alone is undoubtedly due to the precipitation of somatic antigens from the culture filtrate along with the mucinolytic enzyme during the preparation of the solution. Absorption of these sera with washed vibrios reduces the agglutinin titer without affecting the antimucinase activity(7). This, together with the observation that the cholera vaccine (containing non-viable cells) fails to stimulate the production of antimucinase, provides evidence that the mucinase and the somatic antigens of *V. comma* are not immunologically related, thus confirming the findings of Burnet and Stone(1) and Jensen(3).

Discussion. The role of the cholera mucinase in the pathogenesis of Asiatic cholera has not as yet been definitely established by direct evidence. The studies of Burnet and Stone(1) and those of Lam, Mandle and Goodner(2) suggest that this enzyme can induce intestinal damage such as may resemble the effects of cholera in man. However, the report by Singh and Ahuja(8) of mucinolytic activity in culture filtrates of vibrios isolated from non-cholera sources suggests that this enzyme may at best be only one of several

TABLE III. Antimucinase and Agglutinin Titers of Sera of Immunized Rabbits.

Immunizing antigens	Rabbit No.	Anti-mucinase titer	Agglutinin titer
None (pre-immunization sera)	1, 2	<20	<20
	4, 5	<20	<20
	7, 8	<20	<20
Mucinase solution*	1	640	1280
	2	320	320
	3	640	1280
Mucinase solution + cholera vaccine† (1:1)	4	320	640
	5	320	1280
	6	640	1280
Cholera vaccine	7	<20	1280
	8	<20	1280
	9	<20	1280

* Mucinase solution prepared from culture filtrate of *V. comma* strain 20A78.
† Lederle Cholera Vaccine, Lot No. 1976-67K.

factors involved in the disease process. If this proves to be the case, a truly effective cholera vaccine would have to contain a number of antigenic components. Jensen(3) has pointed out that the cholera mucinase must be maintained in its native state if it is to retain its antigenicity. Since the enzyme in the vibrio culture filtrate was extremely unstable, he suggested lyophilization as a method of preserving the *in vitro* activity and antigenicity of this material. The results of the present study, however, indicate that it is possible to obtain a stable mucinase solution, which can be combined with the present fluid cholera vaccine without altering the antibody-stimulating properties of the enzyme or of the somatic antigens.

No experimental data are available at this time to provide an explanation for the stability of the mucinolytic enzyme in the purified preparation as contrasted with the extreme lability of this enzyme in the culture filtrates. The purification procedure may have removed or inactivated some substance, perhaps another enzyme, which is responsible for the rapid deterioration of the mucinase. The possibility that one of the ingredients of the buffered calcium saline solution, employed as the solvent for the purified material, is protective must also be considered since Burnet and Stone found that the enzyme in culture filtrates diluted in buffered calcium saline is less readily inactivated by heat than in undiluted filtrates. Additional studies are planned to elucidate this point.

The partially purified mucinase solution has, in addition to its stability, other features which enhance its value as an immunizing antigen. The use of a dialyzed medium, which is not precipitated by ammonium sulfate, in the preparation of this material has eliminated high molecular weight medium constituents which might cause undesirable reactions when injected into animals or humans, and thus eliminates the necessity of developing chemical fractionation procedures for the removal of these substances. Furthermore, the preparation contains, in addition to the mucinase, other products of vibrio metabolism, some or all of which may be desirable components of an immunizing agent for Asiatic cholera.

Summary. 1. A method is described for the preparation from cholera culture filtrates of a mucinase solution which retains its *in vitro* mucinolytic activity for periods in excess of 2 years. 2. This preparation has been combined with a commercially prepared fluid cholera vaccine without affecting the *in vitro* activity of the enzyme, the antigenicity of the mucinase, or the antigenicity of the vibrio somatic substances. 3. The advantages of employing such a preparation as an immunizing agent for Asiatic cholera are discussed.

The technical assistance of Mr. Gerald A. Cole is gratefully acknowledged.

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Propagation of Measles Virus in a Strain of Human Epidermoid Cancer Cells (Hep-2).* (22677)

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In 1954 Enders and Peebles(1) reported the isolation of a cytopathogenic agent from patients with measles. Human and monkey kidney cultures were used in their study, but they reported that uninoculated monkey cultures may contain transmissible agents which cause effects at times difficult to distinguish from those of measles virus. The infectivity titer of human kidney cultures, including fluid and disrupted cells, was only $10^{3.5}$ TCD_{50} per ml. The purpose of this note is to report the adaptation of measles virus to a human cancer cell strain, Hep-2(2), and certain characteristics of the virus grown in this cell strain. Hep-2 is easily grown in the absence of human serum, has thus far proved free of latent agents, and can yield titers of measles virus greater than 10^6 TCD_{50} per ml.

Methods. Adaptation to Hep-2 cells. The 23rd human kidney passage of the Edmonston strain of measles virus† was inoculated into cultures of several cell strains, including Hep-2.† All cultures were kept in Eagle basal medium for HeLa cells(3) supplemented with 10% calf serum. The medium was changed every 4-7 days. No cytopathic (CP) changes characteristic of measles (multinucleate giant cells with intranuclear inclusions) were observed, but one HeLa culture degenerated in a nonspecific manner after 38 days incubation. When fluid from this culture was passaged into fresh HeLa and Hep-2 cultures, a non-specific type of degeneration again occurred in the HeLa culture but giant syncytial cells were found in Hep-2. Characteristic CP effects have been noted in later HeLa passages but they have been easier to recognize in Hep-2 cultures where the controls can be maintained in excellent condition for weeks in the medium free of human serum. CP changes have been observed in Hep-2 cultures 2 days after infection when large inocula were used but with small inocula 3-4 weeks might pass before recognizable changes appeared. Another measles virus line has been established in Hep-2 without HeLa passage, but after 2 passages in monkey kidney culture and with similarly prolonged initial incubation periods.

Results. Only 6 passages in Hep-2 cells have been made to date but multiplication has been established by the facts that, including changes, a total dilution of about 10^{46} of our original seed material has been involved and the titer of the last harvest was 100 times greater than that of the initial seed. The identity of the virus has been established by its characteristic CP effect and by a neutralization test. The typical CP effect was best manifest when the cultures were grown in Enders biological medium containing 35% Hanks salt solution, 35% beef amniotic fluid, 25% heated horse or calf serum and 5% beef embryo extract, or in a synthetic medium lacking glutamine(4). The neutralization test (Table I) was carried out by mixing 1/10 acute or convalescent serum from patients with measles, with serial virus dilutions and incubating one hour at room temperature before inoculation of Hep-2 cultures. Dr. John F. Enders had found that these patients had developed a marked rise in measles antibodies and kindly made aliquots of the sera available to us.

Growth of Virus in Hep-2 Cells. Cultures of Hep-2 cells in Eagle medium were inoculated with 10^5 TCD_{50} of adapted measles virus and washed 2 hours later. New virus was first detectable 18 hours after inoculation when the cells were ruptured by freezing and

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† The stock of measles virus and acute and convalescent sera were graciously supplied by Dr. John F. Enders of the Children's Hospital, Boston. The original stock of Hep-2 and of certain other cultures which were tested, were obtained through the kindness of Dr. Alice E. Moore of the Sloan-Kettering Institute, New York City.

TABLE I. Neutralization of Hep-2 Passage Measles by Human Serum.

Serum	Virus titer (neg. log.)	Logs virus neutralized
None	7.0	
Acute	7.2	0
Convalescent	2.5	4.5

thawing. Free virus first appeared in the fluid about 12 hours later (Table II). Peak titers were achieved in 2½ days. The maximum complement-fixation titers have been ⅛. Enders and Peebles(1) reported that their human kidney grown material provided a usable antigen without concentration. It is thus apparent that Hep-2 cultures do not offer, in comparison with human kidney, proportionally as great increases in C-F antigens as of infective virus.

Infection of monkeys. Enders(5) has found the rhesus and cynomolgus macaques may possess naturally acquired antibodies against measles virus. In an attempt to determine how widespread this phenomenon might be, sera from 10 monkeys of various species have been tested. Six of these, including rhesus (*Macaca mulatta*), mangabey (*Cercocebus torquatus lunulatus*), green (*Cercopithecus aethiops sabeus*) and African white (*C. a. tantalus*), possessed virus inhibitors in their serum. The same sera, as well as 4 specimens from the African red grass monkey (*Erythrocebus patas*), gave positive C-F reactions with measles antigen. Eight monkeys, including 4 rhesus without pre-existing neutralizing antibodies, were inoculated intramuscularly or intravenously with 1 ml of Hep-2 passage virus. One of those with preantibodies had an elevated temperature and bouts of shivering for 4 days, beginning 12 days after injection. It is questionable whether this illness was associated with the inoculation. The only sign of illness in the other monkeys was a marginal temperature elevation in one monkey without antibodies 13 days after inoculation. Sera from 4 monkeys with antibodies and 2 without were checked for viremia. Positive results were obtained 7 and 13 days after inoculation from those monkeys without prior antibodies but not from the others. The virus that was isolated from the monkeys' blood did not cause recognizable CP changes in Hep-2 cells until after 30 to 56 days of incubation. It seems possible that the single monkey passage had partially reversed the adaptation of the virus to Hep-2 cultures.

TABLE II. Growth of Measles in Hep-2 Culture.

Hr after inoculation	Virus titer (neg. log.) Frozen cells	Supernatant fluid
12	0	0
18	3.0	0
30	3.7	1.2
57	6.0	5.2

Summary. Measles virus has been adapted to a cancer cell strain derived from a human epithelioma. The Hep-2 cell strain provides a particularly useful system for the study of measles because of its growth characteristics in media free of human serum, its freedom from latent cytopathogenic agents, and the high yields of virus that may be rapidly obtained. After adaptation to Hep-2 the virus was still capable of infecting monkeys but caused little or no illness.

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Hypoglycemic Action of Indole-3-Acetic Acid by Mouth in Patients with Diabetes Mellitus.* (22678)

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The administration of indole-3-acetic acid by mouth to normal rats results in a statistically highly significant hypoglycemia and a concomitant reduction in the rate of insulin destruction.† Since a similar decrease in the blood sugar does not occur in the severely diabetic alloxanized rat, the response cannot be due to an inhibition of the secretion or action of glucagon or to an acute non-specific hepatotoxic action. Accordingly the hypoglycemic response must be dependent upon the presence of insulin. This insulin dependence may be due to either an increase in the secretion or to a decrease in the rate of destruction of endogenous insulin consequent to an inhibition of the enzyme system, insulinase, which is relatively specific in catalyzing the hydrolysis of insulin. In accord is the fact that indole-3-acetic acid is an inhibitor of insulinase *in vitro* and *in vivo*.†

The present report deals with observations in man on the acute action of indole-3-acetic acid by mouth on the blood sugar concentration.

Method. Twelve adult patients with diabetes mellitus and 6 subjects without any evidences of a disturbance in carbohydrate metabolism were studied. In all instances, the patients with diabetes mellitus developed clinical evidences of the disorder after the age of 40 years (mean age of onset $\pm$ SE $=$ 49.2 $\pm$ 2.0 years). The duration of the syndrome was from 2 to 16 years (mean duration $\pm$ SE $=$ 7.7 $\pm$ 1.3 years). Those patients who were maintained on long-acting insulins were transferred to regular insulin for 3 to 5 days before the study in order to obviate the effect of insulin depots. All subjects served as their own controls in that the effect of drinking 5 ml of 0.5% sodium bicarbonate, (pH 7.8)/kg body weight on the blood sugar concentration was studied on one occasion and the effect of a similar volume of bicarbonate containing 100 mg indole-3-acetic acid/kg body weight was determined on another occasion. The subjects were fasted overnight before each test and those with diabetes were not given any insulin on the morning of the test. Venous blood samples were taken before and at hourly intervals for 5 hours after the patients drank the solution and the concentration of glucose was determined by Nelson's method(1).

Results. A small, but statistically significant decrease in the blood sugar concentration occurred in the apparently healthy subjects after the ingestion of the indole-3-acetic acid dissolved in sodium bicarbonate (Fig. 1). Whereas the blood sugar concentration (mean $\pm$ SE) showed a slight increase from 82.7 $\pm$ 5.8 to 93.5 $\pm$ 5.8 mg % by the end of the 5 hours after the ingestion of the sodium bicarbonate, the blood sugar decreased from 81.7 $\pm$ 4.0 to 68.6 $\pm$ 2.7 mg % during the same interval after the indole-3-acetic acid solution. By the third hour the difference between the 2 responses was statistically highly significant ($P<0.001$).

The patients with diabetes mellitus likewise developed a hypoglycemic response to the indole-3-acetic acid (Fig. 2). Whereas these patients developed a maximum decrease in the blood sugar concentration from 257.3 $\pm$ 10.2 to 210.5 $\pm$ 13.3 mg % in 5 hours after the ingestion of the sodium bicarbonate solution, the blood sugar decreased from 232.9 $\pm$ 16.0 to 143.4 $\pm$ 11.8 mg % during the same interval after indole-3-acetic acid. The difference between the responses to the two solutions was statistically significant by the second hour ($P>0.01$) and thereafter. A repeated measures analysis of variance(2) likewise revealed a significant difference ($P>0.01$) between the curves of the blood sugar

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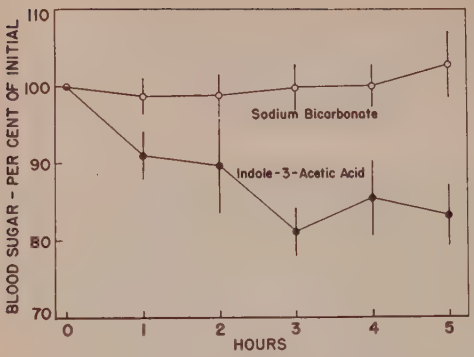

FIG. 1. Effect of indole-3-acetic acid on blood sugar concentration of non-diabetic subjects. Blood sugar concentration at each interval is expressed as the % (mean ± S.E.) of concentration prior to ingestion of the solution. Initial blood sugar concentration before the sodium bicarbonate solution was 82.7 ± 5.8 mg %, and 81.7 ± 4.0 mg % before the indole-3-acetic acid solution.

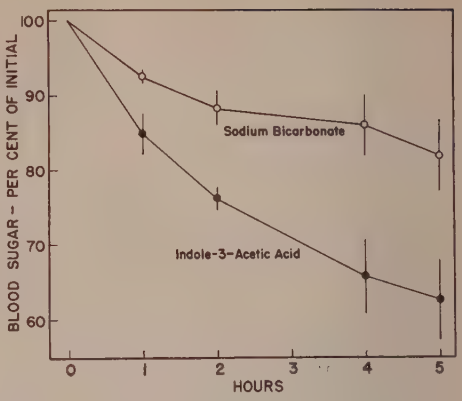

FIG. 2. Effect of indole-3-acetic acid on blood sugar concentration of patients with diabetes mellitus. Blood sugar concentration at each interval is expressed as % (mean ± S.E.) of concentration prior to ingestion of the solution. Initial blood sugar concentration before the sodium bicarbonate solution was 257.3 ± 10.2 mg %, and 232.9 ± 16.0 mg % before the indole-3-acetic acid solution.

responses to the two solutions.

Discussion. The relatively gradual decrease in the blood sugar concentration after the ingestion of indoleacetic acid by the non-diabetic subjects differs from the rapid maximal hypoglycemia which results within one hour after 1-butyl-3-p-tolylsulfonylurea by mouth(3). Various considerations have led to the hypothesis that the immediate hypoglycemic response of non-diabetic subjects to the tolylsulfonylurea is due to the discharge of insulin from the Islets of Langerhans into the circulation while the persistence of the hypoglycemia is due to the inhibition of insulinase and a consequent decrease in the rate of destruction of endogenous insulin(3). The response of the non-diabetic subjects to the indoleacetic acid, however, suggests that an acute stimulation of the Islets of Langerhans does not occur after the ingestion of this compound. Both the diabetic and non-diabetic groups show a gradual decrease in the blood sugar concentration which is similar to that observed in responsive patients with diabetes after the administration of tolylsulfonylurea (4) and may be attributed to an inhibition of insulinase and a decrease in the rate of insulin destruction. The data are in accord with the hypothesis that the insulin insufficiency of many patients with diabetes mellitus is due to an increase in the rate of destruction of insulin by the tissues rather than to a marked decrease in the rate of production of insulin by a severely damaged pancreas(5).

Although the data reported herein suggest that indoleacetic acid can be effective by mouth in decreasing the blood sugar of patients who developed diabetes after 40 years of age, the relatively small number of patients studied and the lack of information concerning the long-term toxic effects of indoleacetic acid and other plant growth hormones precludes their application to the therapy of diabetes mellitus at the present time. The studies, however, do suggest an hitherto unsuspected relationship between plant and animal hormones.

Conclusions. Indoleacetic acid by mouth produces a decrease in the blood sugar of adult patients with diabetes mellitus.

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Effect of Castration upon Susceptibility to MEF_1 Poliomyelitis Virus Inoculated Intraperitoneally in Hamsters.* (22679)

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It has been shown that in comparison with normal hamsters inoculated intracerebrally with MEF_1 virus, the castrated animals showed a higher mortality from poliomyelitis while those treated with testosterone or gonadotrophic hormone showed a lower mortality(1). In order to determine whether castration is able to overcome the refractory state of hamsters to intraperitoneal inoculation of virus, the susceptibility to this route of inoculation was investigated at various periods following castration.

Materials and methods. Male Golden hamsters, 25-50 g, were used. A volume of 0.5 ml MEF_1 virus suspension diluted 1/50 in saline (*i.e.*, 800 Mouse LD_{50}) was inoculated intraperitoneally. Bilateral orchiectomy was performed at different intervals before and after inoculation of virus and infectivity in castrated animals was compared with that of non-castrated controls. All paralyzed animals were sacrificed and, in order to confirm the clinical diagnosis, their spinal cords were examined histologically and a suspension of the cord was inoculated into mice. Cortisone (1.5-2.5 mg) in a single intramuscular administration was injected for the study of individual and combined effects of castration and cortisone treatment. To investigate possible protective effects associated with compensatory phenomena following castration, the refractory state to intraperitoneal inoculation was eliminated by means of a single intramuscular injection of cortisone immediately prior to the viral inoculation. The results were analyzed by statistical procedures previously described(1).

Results. Seventeen % of castrated hamsters receiving MEF_1 virus intraperitoneally, 24 hours following orchiectomy, developed paralytic poliomyelitis, confirmed by mouse inoculation and by histological examination of the spinal cord. None of the similarly inoculated but uncastrated control animals developed the disease (Table I). When castration was performed 3 days after the administration of virus, only 1 animal out of 45 developed the disease, which may be explained by our previous observation of the rapid disappearance of intraperitoneally injected virus(2). Recent castration plus small doses of cortisone revealed a synergistic effect in enhancing susceptibility to poliomyelitis virus as compared to either castrated or cortisone treated controls alone (Table I).

The findings with respect to intraperitoneal inoculation of virus 14 days after orchiectomy were similar to those previously reported in hamsters inoculated intracerebrally where it was shown that compensatory phenomena usually occur at that stage following castration substituting a degree of resistance for the initial enhancement of susceptibility induced by testicular suppression(1). To determine whether a similar protective effect may be observed 14 days after castration in peritoneally inoculated animals, 2.5 mg of cortisone was administered intramuscularly followed by MEF_1 virus (800 mouse LD_{50}) inoculated intraperitoneally the same day. A significantly lower proportion of 14-day castrates developed the disease as compared to both 24-hour castrates and uncastrated controls receiving the same dose of cortisone (Table I).

Overdosage of testosterone significantly diminished the mortality of cortisone treated hamsters inoculated intraperitoneally with MEF_1 virus (Table II). However the reduction in paralytic incidence and mortality was of little statistical significance if the animals dying without paralytic symptoms were eliminated from both treated and control groups (Table II).

Discussion. From the above experiments it may be observed that orchiectomy is a conditioning factor in the pathogenesis of experi-

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TABLE I. Effects of Castration and of Cortisone Treatment upon Infectivity by Peritoneal Route in Hamsters. (All groups received a single intraperitoneal inoculation of MEF_1 virus, 800 mouse LD_{50}.)

Groups	Cortisone, mg	No. animals	Paralytic incidence, %	Significance of difference "P"
Non-castrated control	0	60	0	.001
24 hr after castration	0	35	17	
Idem	.5–1.0	58	21	.001
"	1.5–2.5	63	54	
Non-castrated control	1.5–2.5	105	36	.01
14 days after castration	2.5	29	10	

mental poliomyelitis since it enhances susceptibility to intracerebrally inoculated virus(1), and overcomes the refractory state to poliomyelitis virus inoculated peritoneally. With time, however, compensatory mechanisms come into play, and a state of resistance follows initial susceptibility induced by orchiectomy. This biphasic effect of castration raises the question as to whether susceptibility alterations are the result of suppression of testicular function or whether they are secondarily induced by the adrenal alterations that follow orchiectomy. It has been shown that during the initial 5 day period following castration, adrenal hypertrophy and involution of the thymus coincide with enhanced susceptibility to MEF_1 virus, while 14 days later, a progressive adrenal involution and thymus hypertrophy coincide with increased resistance to poliomyelitis(1). The fact that both adrenal hypertrophy and enhanced susceptibility to poliomyelitis follow any traumatic stress(2), suggests that during the initial stage following castration, enhancement of susceptibility was produced as a consequence of operatory trauma rather than by the suppression of testicular function. However, several other observations such as the inverse relationship between testicular maturity and susceptibility to poliomyelitis in relation to age and seasonal fluctuations(3), the protective effect of gonadotropine and of testosterone treatment(1), the association of large testes and resistance to poliomyelitis infection in certain races of hamster (Chinese, Albino), would indicate that testicular hormones have a protective influence against experimental poliomyelitis. Actually it is not possible to determine the individual contributions of adrenal gland and testis in determining the susceptibility level, since in addition to the possible direct hormonal interactions, the two endocrines influence each other indirectly through the mediation of the pituitary gland. Thus, orchiectomy produces polyphasic adrenal alterations which are followed by polyphasic fluctuations in susceptibility to poliomyelitis, while cortisone treatment produces testicular involution which is paralleled by enhancement of both morbidity and mortality from poliomyelitis(1). When cortisone treatment follows castration, their combined effects may be synergistic (initial stage), or

TABLE II. Effect of Testosterone Overdosage upon Mortality in Hamsters Injected with 3 mg Cortisone Intramuscularly and with MEF_1 Virus Intraperitoneally (800 Mouse LD_{50}).

		Total mortality (including animals dying without paralytic symptoms)		Mortality from poliomyelitis (excluding animals dying without paralytic symptoms)			
Preliminary treatment	No. of animals	Mortality, %	Significance "P"	Mortality, %	"P"	Paralysis, %	"P"
Depo–Testosterone* (15 mg i.m.)	249	43	.0002	29	.03	40	.04
Control	245	60		40		51	

* Upjohn Co.

antagonistic (compensatory stage), depending upon the experimental timing. From the effects of these interacting mechanisms it may be concluded that: refractoriness may be converted into susceptibility to poliomyelitis and vice versa, by factors influencing the adrenal-testis equilibrium either by direct action or by interference with pituitary function. The latter mechanism, which seems to be responsible for the effects of certain seasonal and dietary factors upon susceptibility(2,3), should be taken into consideration as one of the prospective means for disease prevention.

Summary. 1. A significant proportion of castrated male hamsters inoculated with MEF_1 virus intraperitoneally developed paralytic poliomyelitis, whereas non-castrated controls were completely refractory to the virus by this route. 2. Enhancement of susceptibility observed immediately after castration was found to be reversed during the compensatory stage 2 weeks later. 3. The effect of castration upon susceptibility to experimental poliomyelitis was synergistic with cortisone during the initial stage, but antagonistic during the compensatory stage.

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Relationship between Behavioral Changes and Brain Cholinesterase Activity Following Graded Intracerebral Injections of DFP. (22680)

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Previous studies have shown that forced circus movements may be induced by unilateral electrostimulation(1,2,3,4) or ablation (5,6,7,8) of various parts of the neuraxis. Forced circling may also be produced by unilateral intracarotid injections of the anticholinesterase diisopropylfluorophosphate (DFP)(9,10,11,12) as well as by micrometer syringe injections of DFP and CNS stimulants into specific brain structures.† It has been disclosed that the intracerebral administration of 0.01 ml solutions containing from 0.06 to 0.16 mg of DFP does not result in significant spread of this substance to adjacent cerebral gray matter.† This finding opened the possibility to determine (1) the level of cholinesterase (ChE) activity at which stimulation of specific CNS structures occurs and (2) the relationships between the dose of DFP, the level of ChE activity and the production of forced circling. The caudate nucleus was selected for this study because of its comparatively high level of ChE activity(10,12) and the known role it plays in the forced circus movements induced by micrometer injections into this structure.†

Methods. In all experiments DFP was administered into the right caudate nucleus by means of a micrometer syringe attached to a stereotaxic apparatus. Solutions of DFP were made by diluting weighed amounts with water so that injections of 0.01 ml would deliver between 0.0001 mg to 0.32 mg. In order to inject greater doses undiluted quantities of DFP were employed because DFP is immiscible in this volume of water in amounts greater than 32 mg. The dose of undiluted DFP was determined by appropriate adjustment of the micrometer syringe and in all such cases the volume was less than 0.01 ml. The entire dose range of DFP employed was from 0.0001 mg to 10 mg. Controls were obtained by injections of 0.01 ml of physiological saline. Mock injections were also made in order to determine whether DFP was de-

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† In abstract, *Fed. Proc.*, 1956, v15, 199.

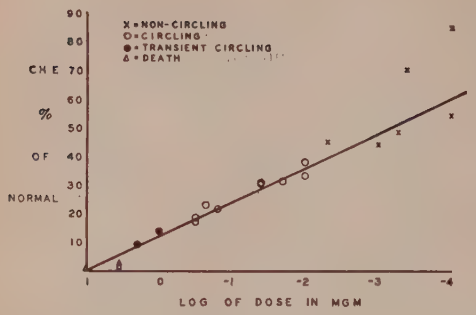

FIG. 1. Relationship between dose, % ChE activity and the behavioral response to inj. of from 0.0001 to 10 mg of DFP into the right caudate nucleus. Circling was always contraversive (*i.e.*, away from the inj. side). Note that one experimental value is shown at naught ChE activity. Also observe that 2 values for circling animals are almost superimposed.

posited in the brain merely upon insertion of the needle. To assure the delivery of solutions to exactly the point desired the bevel of a 27 gauge needle was ground off. This caused the direction taken by the drop to be parallel to the long axis of the needle. The needle was cleared with a stylus before each injection. Animals which had shown signs of bleeding during the experiment or at autopsy were discarded. Only male albino rabbits of approximately the same weight (average 2238 g) were selected for this study so that their cranial dimensions would be as uniform as possible, *e.g.*, the Bregma to Lambda skull length in all of these animals was close to 18.35 mm. A local anesthetic (0.5% pontocaine) was administered subcutaneously over the dorsum of the skull and the scalp was laid open. The animals were then lightly etherized and a hole drilled through the cranium with a dental burr 2 mm anterior to the transverse suture and 2 mm lateral to the longitudinal suture. The injection into the caudate nucleus was made at this point at a depth of 9 mm from the external skull surface. Ether administration was then stopped and the animal was released from the stereotaxic instrument and observed for at least 1 hr. The animals were then sacrificed by an intravenous injection of air and the ChE activities bilaterally determined in the caudate nuclei, dorsomedial thalami and motor cortices by the titrimetric method of Aprison, Nathan and Himwich(10). All ChE determinations were run "blind." In a second series of experiments 0.2 mg of atropine sulfate was administered with 0.16 mg of DFP and in a third one 1 mg of atropine sulfate was injected i.v. after forced circling was induced by the deposition of 0.16 mg of DFP into the nucleus caudatus.

Results. Injections of from 0.01 to 0.32 mg of DFP in solution into the caudate nucleus resulted in every case in contraversive forced circus movements which persisted for longer than 15 minutes (range from 18 min. to 1 hr. and 44 min.). As seen in Fig. 1 the ChE activity of the right caudate nucleus was below 40% of the control value in each of these circling animals. The ChE activity determinations on the left caudate nucleus, however, revealed values which were higher than 50% of normal and in general with doses of from 0.01 to 0.16 mg there was no significant evidence of spread of the injected DFP either to the opposite caudate or to the rest of the brain. With greater doses (0.23 to 0.32 mg) a spread of this substance throughout the brain was evident. Nevertheless the ChE activity values were always more than 50% of the control in parts of the brain other than the right nucleus caudatus. No other effects of DFP such as miosis or head nystagmus(9,11) were observed as a result of the intracerebral injection of this substance.

With doses of 1 and 2 mg of undiluted DFP contraversive circling was less than 8 minutes in duration and only intermittent. Doses of 3 and 10 mg did not cause circling and resulted in the death of the rabbit within 7 to 60 minutes after the intracerebral injections. In all of these animals the ChE activity in the left caudate nucleus was less than 35% of normal and in those that died it was near zero; the other brain areas in fatal cases had decreased to less than 13% of normal values.

On the other hand, injections of less than 0.01 mg of DFP did not cause any apparent change in the behavior of the animal and the ChE activity values were greater than 40% of normal. As shown in Fig. 1 the distribu-

tion of ChE activity of the right caudate nucleus in this noncircling group was greater than with the circling animals. No significant ChE activity reduction (less than 4%) resulted from the mock injections.

Addition of 0.2 mg of atropine sulfate to the injected solution of DFP prevented the appearance of forced circus movements. Moreover, the i.v. administration of 1 mg of atropine was effective in stopping the circus movements induced by previous intracerebral injections of DFP (0.04 to 0.16 mg).

Discussion. The results indicate that the optimum dose for the production of contraversive circling by intracerebral administration of DFP into the caudate nucleus is between 0.01 and 0.32 mg and that no significant spread of DFP from such injections may be expected with doses from 0.01 to 0.16 mg. Greater concentrations lead to an increasingly greater degree of spread of this substance, a finding which suggests that beyond a certain dose the quantity lost is increased progressively either by way of the circulation or cerebral spinal fluid. Because of this spread it was not possible to limit the reduction of ChE activity to a single brain structure to near zero by this method. Such low values for the caudate nucleus, however, have been reported when DFP is administered by means of the common carotid artery whereas other areas of the brain do not suffer a similar profound decrease in enzyme activity(10,13). Under these conditions electrographic(12,14) and other evidence(7) has been advanced to show that the nucleus caudatus is physiologically active. Since it was necessary to excise the entire caudate nucleus in order to obtain enough tissue for analysis, it is possible that the ChE activity of the caudate nucleus at the injection site was more reduced than reported in this study for circling animals. On the other hand, when DFP is administered intracarotidly the lenticulostriate vessels would cause a more uniform distribution of this substance to the caudate nucleus(12) and consequently an analysis of this whole structure may lead to lower values than was obtained by intracerebral injections.

This study also indicates that the ChE activity of one of the two caudate nuclei must be reduced to at least 40% of control value if circling is to be expected. There is no reason to believe that other brain areas need be affected by DFP to induce circling by intracerebral injections into this structure (see also reference†). The similarity between forced circus movements obtained in this study and those resulting from vestibular excitation with DFP(15) suggests a functional relationship between these two systems as proposed by Muskens(16). That death occurs when the total brain ChE activity is 10% of normal or less has been previously reported(17). Interestingly it occasionally took as long as an hour for massive doses of DFP to cause death when injected by micrometer syringe. This prolonged period would seem to offer a special advantage in the study of nerve gas antidotes when lethal doses are employed.

Atropine has been shown to correct the forced circus movements induced by DFP (injected via common carotid artery)(9) and it is thought therefore that DFP whether injected intracarotidly or intracerebrally has its effects by a cholinergic mechanism. The finding that intracerebral injections of DFP may induce circling in the rabbit supports the conclusions of other investigators that DFP administered intracarotidly causes circling through its central effects(13). None of the observable peripheral signs of the adversive syndrome(9,11) such as ipsiversive miosis are evident with intracerebral injections of DFP. The similarity between responses obtained with electrostimulation of different brain areas and micrometer syringe injections of DFP† reveal the possibility that this last procedure may be used as an additional method to study experimentally CNS cholinergic drugs and their antagonists.

Summary. The relationship between the amount of DFP injected into the right caudate nucleus and the resulting decreased cholinesterase activity has been investigated in regard to the appearance of forced circus movements. DFP in doses of less than 0.01 mg had no observable behavioral effect and did not lower the ChE activity to 40% of the control value. Doses of from 0.01 to 0.16

mg, however, caused persistent contraversive forced circling and decreased the cholinesterase value of the nucleus caudatus to from 21 to 40% of normal. There was no evidence of significant spread of the DFP within the brain in this group of animals. The administration of atropine sulfate either prevents or stopped these forced circus movements. Persistent forced circling could also be induced with larger doses of from 0.23 to 0.32 mg of DFP and evidence of spread of the DFP was then observed. In this group the injected nucleus caudatus showed ChE activity values of from 18 to 20% of the control whereas remaining parts of the brain revealed symmetrical reduction in ChE activity between 48 to 82% of normal. When still greater amounts of DFP were injected (1 to 10 mg) the spread of this substance progressively increased so that eventually ChE activity was decreased bilaterally and transient circling or death resulted.

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Sensitivity of *Staphylococcus aureus* to Penicillin in Various Media. (22681)

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The composition of the culture medium is of importance in testing the sensitivity of microorganisms to penicillin. The media may contain substances which react with penicillin chemically, thereby reducing its effective concentration. Substances having —SH groups such as thioglycollate, cysteine and glutathione behave in this manner(1,2,3,4). Acetic acid, too, inactivates penicillin by chemical interaction(5). Vitamins such as folic acid and vit. B_6 antagonise the effect of penicillin (6), and ascorbic acid also shows a deleterious effect(7). Glucose may diminish the effect of penicillin when added to cultures of *Staphylococcus aureus*(8,9). Furthermore, the activity of penicillin was decreased by addition of serum to the basal medium(10). On the other hand, certain substances, such as nicotinamide, exhibit synergistic effects with penicillin(8,11).

The present work deals with the effects of the addition of various carbohydrates to culture media, on the sensitivity of *Staph. aureus* to penicillin.

Methods. Test organism and inoculum: *Staphylococcus aureus* Oxford strain, was inoculated into nutrient broth (Difco). After 20 hours incubation at 37°C the cells were harvested and washed 3 times with physiological saline. The washed cells were diluted 1:100 in saline and 0.1 cc added to tubes containing 1 cc of medium. Two different media were employed: (a) Nutrient broth (Difco) and (b) a casein hydrolysate medium of the following composition:

Casein hydrolysate (vit. free)* 10%	3 cc
Carbohydrate	1000 mg
Tryptophan	20 mg
Cystine	10 mg
Thiamine	100 γ
Nicotinic acid	100 γ
Distilled water to make 100 cc	

* Obtained from Nutritional Biochemicals Co. (NBC).

After autoclaving, the following sterile solutions were added in amounts of 0.5 cc each: 1) Salt solution composed of: $MgSO_4 \cdot 7H_2O$ 4%; NaCl 0.125% $FeSO_4 \cdot 7H_2O$ 0.125%, and $MnSO_4 \cdot 4H_2O$ 0.125%. 2) K_2HPO_4 8.7%, and the medium was dispensed aseptically, into sterile tubes (12 x 100 mm).

Penicillin solution: Crystalline penicillin G (Shenley) was used. A concentrated solution containing 10,000 u/cc was kept in the refrigerator for one week; dilutions being prepared freshly for each experiment.

Antibacterial assay. The assays were carried out in small test tubes containing 1 cc of the medium. The penicillin effect was tested by the 2-fold dilution technic using the medium as a diluent for the penicillin. After inoculation with *Staph. aureus* the tubes were incubated at 37°C for various periods of time.

Results. Effect of different carbohydrates *on the sensitivity of Staph. aureus to penicillin*. Nutrient broth and casein hydrolysate media, containing glucose, pyruvic acid, acetic acid or lactic acid were inoculated with *Staph. aureus*. (Carbohydrates were added to the media before autoclaving.) Controls without added carbohydrate were included. Penicillin was added to the tubes in various concentrations, and the degrees of growth inhibition were examined after incubation for 24, 48, 72, and 96 hours. The results are given in Fig. 1, which shows that *Staph. aureus* was most sensitive to penicillin in nutrient broth and in a casein-hydrolysate medium containing pyruvic acid; 0.04 u/cc of penicillin inhibited the growth completely. The degree of inhibition did not change appreciably on further incubation. On the other hand, 0.2 u/cc of penicillin was not inhibitory when a casein hydrolysate medium without added carbohydrate was used. When lactic acid was added to casein hydrolysate, growth appeared in tubes containing 0.2 u/cc only after 72 hours of incubation. The growth of *Staph. aureus* in a medium containing acetic acid was not inhibited by concentrations of 0.15 u/cc after 96 hours. In Fig. 1 the sensitivity of *Staph. aureus* is plotted against incubation time. The straight line obtained in a glucose and penicillin-containing medium suggests the occurrence of a chemical interaction between them. To examine this point further, the following experiment was performed: penicillin in a concentration of 5,000 u/cc was incubated at 37°C in a solution of glucose (50 mg/cc). Penicillin incubated similarly in

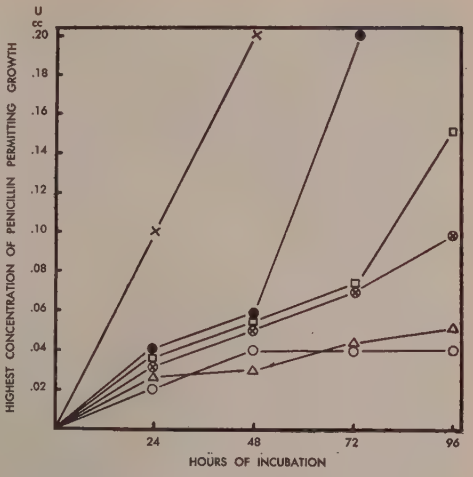

FIG. 1. Effect of carbohydrates on inhibition of *Staphylococcus aureus* by penicillin. Casein hydrolysate medium, —×—; casein hydrolysate medium with lactic acid (1%), —●—; casein hydrolysate with acetic acid (1%), —□—; casein hydrolysate with glucose (1%), —× (in circle)—; casein hydrolysate with pyruvic acid (1%), –△–; nutrient broth (Difco), —○—.

A B

FIG. 2. Effect of glucose on inhibition of *Staphylococcus aureus* by penicillin. (A) Penicillin (5000 u/cc) was incubated with glucose (50 mg/cc) for 5 days at 37°C. Sterile filter paper strips were soaked with the solution and tested for activity against *Staphylococcus aureus*. Photographs taken after growth for 48 hr. (B) Penicillin (5000 u/cc) was incubated with saline solution for 5 days at 37°C. Sterile filter paper strips were soaked with the solution and tested for activity against *Staphylococcus aureus*. Photographs taken after growth for 48 hr.

saline served as a control. After 5 days, pieces of sterile filter paper were soaked in the respective solutions and were placed on agar plates inoculated with *Staph. aureus*. The results are shown in Fig. 2. It is seen that on incubation of penicillin with glucose a marked decrease of the inhibition zone was obtained. No diminution of penicillin activity was found after it was preincubated with lactate; it seems unlikely that a similar chemical reaction between penicillin and lactate had occurred. To eliminate the possibility that the addition of lactic acid stimulated the occurrence of a resistant mutant, the lactate-grown strain was inoculated into nutrient broth and its sensitivity to penicillin re-examined; however, no diminution in sensitivity was observed. Parallel experiments in which the media were sterilized by Seitz filtration were carried out. Table I shows that heating is not the cause of the variation in sensitivity in the different media.

Discussion. Pyruvic acid and glucose when added to media rich in amino acids (supplied as casein hydrolysate), increase the sensitivity of *Staph. aureus* to penicillin. On the other hand, lactic and acetic acids were not effective. The observed interaction between penicillin and glucose explains the reduction in penicillin activity after prolonged incubation in glucose containing medium. No such mechanism is responsible for the low activity of penicillin in lactic acid-medium. Potentiation of the penicillin activity by pyruvate may be explained as follows: (1) The antibiotic interferes with some step in the pyruvic acid metabolism of *Staph. aureus;* in the absence of added pyruvic acid such interference is less pronounced. Or (2) The toxic factor responsible for the antibacterial effect is *not* penicillin *per se* but a compound formed under its influence(12); pyruvic acid contributes to the production of the hypothetic toxic factor. In the absence of pyruvic acid the inhibitor is formed in smaller amounts, hence the lower antibacterial activity. Although the experiments presented do not favor one explanation rather than the other, unpublished work(13) suggests that penicillin interferes with the oxidation of pyruvic acid by cells of *Staph. aureus*. These results indicate that the sensitivity of *Staph. aureus* to

TABLE I. Sensitivity of *Staphylococcus aureus* to Penicillin in Casein Hydrolysate Media Containing Various Carbohydrates.*

	Acetic acid	Lactic acid	Pyruvic acid	Glucose	Nutrient broth	Casein hydrolysate, no carbohydrate (control)
Autoclaved medium	.15†	.2	.025	.1	.025	>.2
Seitz filtered medium	.15	.2	.05	.1	.025	>.2

* Results were read after 96 hr of incubation at 37°C.
† Maximal concentration of penicillin (u/cc) in which growth appeared.

penicillin may be affected by the carbon source of the medium. Similar findings were reported for *E. coli*(14) which was found to be more sensitive to penicillin in the presence of xylose or ribose than with glucose.

Summary. The effect of the composition of the medium on the sensitivity of *Staphylococcus aureus* to penicillin was tested. Pyruvic acid or glucose, when added to a casein hydrolysate medium, increase the antibacterial effect of the antibiotic. Lactic or acetic acids, on the other hand, have no such effect. The possible mechanisms of the enhanced penicillin activity in presence of pyruvic acid are discussed.

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Metabolic Studies of Desaminothyroxine. (22682)

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Desaminothyroxine (3,5 diiodo-4 (3′, 5′, diiodo-4 hydroxy phenoxy) phenyl-propionic acid) was synthesized by Clayton, *et al.*(1) at the Glaxo Laboratories, Middlesex, England, and by Kharasch *et al.** at the University of Southern California.

Bruice, Winzler and Kharasch(2) reported that in tadpoles (*Rana catesbiana*) at about 24°C, desaminothyroxine possesses 130 times the metamorphosing activity of L-thyroxine. Roth(3) has recently reported that it has 1000 times the activity of L-thyroxine in this test at 16°C. In comparison, Roche(4) has shown desaminothyroxine to have about 75% the antigoitrogenic activity of D-L-thyroxine in rats. Selenkow and Asper(5) reported it to have only 20 to 25% the antigoitrogenic activity of L-thyroxine. No reports on its calorigenic activity in mammals have been found.

* Personal communication.

Method. Measurements of the oxygen consumption of rats were conducted in a 5-unit basal metabolic rate machine at 30°C(6). Individual studies usually lasted 4½ hours, using the first half-hour as a calibration pe-

TABLE I. Comparison of O_2 Consumption of Desaminothyroxine on Na L-Thyroxine.

Drug	Body wt, g	Initial body wt, baseline	O_2 consumption (g O_2/100 g rat/hr) 3 days	7 days	10 days	Avg increase O_2 consumption at 10 days (g O_2 /100 g/rat/hr)
		A. Dosage—.025 mg/day				
Desaminothyroxine	210 ± 50	.141	.155	.172	.186	.045
Na L-thyroxine	200 ± 30	.143	.201	.214	.218	.075
Controls	195 ± 25	.136	.127	.138	.144	
		B. Dosage—.075 mg/day				
Desaminothyroxine	215 ± 55	.141	.161	.158	.194	.053
Na L-thyroxine	200 ± 30	.143	.230	.262	.275	.132
Controls	190 ± 10	.135	.135	.135	.143	

riod on rats fasted 24 hours. Four control runs were performed on each of 10 Long-Evans rats, weighing 160 to 230 g. The average oxygen consumption was .142 g O_2/100 g rat, per hour. The studies were carried out under conditions similar to those obtained by other investigators(8). Each animal acted as its own control and another control animal was evaluated with each group to check the variations of the machine. Four rats were injected intraperitoneally with coded Na-L-thyroxine, 0.025 mg/day; and four rats were given intraperitoneal injections of desaminothyroxine, 0.025 mg per day, in a double blind procedure. Individual studies were made at 3- to 4-day intervals for 2 weeks, reaching maximum oxygen consumption at 10 days. At this dosage the desaminothyroxine was found to be 60% as active as L-thyroxine, measured by the increase of oxygen consumption computed as grams of oxygen per 100 g of rat/hour. The 2 groups were then injected intraperitoneally with 0.075 mg daily, per rat, of desaminothyroxine, or L-thyroxine, for 2 weeks under the double-blind procedure. After 10 days on the tripled dosage, desaminothyroxine had only 40% the oxygen consumption of L-thyroxine. It was also noted that this drug produced only a 40% increase of oxygen above the control level, as compared to a 100% increase produced by L-thyroxine. (Table I).

Results. In trial studies at dosages of 0.15 and 0.30 mg daily/rat, desaminothyroxine was shown to have about 30% the calorigenic activity.

All animals returned to the pretreatment oxygen consumption levels after cessation of the drugs. No toxic signs or symptoms were noted during the one month course of drug administration, or in the one month following cessation of the medications. One rat died because of machine failure. The observation of Freinkel, *et al.*(9) of the marked absorption of thyroxine by glassware was confirmed. All drug containers were siliconized.

Discussion. The results, though variable with respect to the dosage, show that the metabolic effect of desaminothyroxine is of the same magnitude as that found in studies of its antigoitrogenic activity. In this respect the drug is less potent than L-thyroxine, but in comparison with the process of metamorphosis in amphibians, there is a wide variance (130 times more potency than Na L-thyroxine at 24°C(3) to 1000 times at 16°C)(4). These effects suggest the multiple activities of these drugs; perhaps separate or multiple metabolic pathways are involved.

Summary. Desaminothyroxine was shown to have 60% of the calorigenic activity of L-thyroxine at dosages of 0.025 mg/rat/day; 40% at the dosage of 0.075 mg/rat/day; and about 30% at dosages of 0.150 and 0.300 mg/rat/day.

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Inhibitory Effect of Chlorpromazine on Arterial Lipid Deposition in Cholesterol Fed Rabbits.* (22683)

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Reduced arterial lipid deposition in cholesterol fed rabbits has been achieved in a number of ways(1). Most of these inhibitory procedures cause weight loss, debilitation or other injurious effects. Chlorpromazine can be administered to animals for long periods of time without causing apparent impairment of health. Nevertheless, it is said to depress cellular activity, lower body temperature and arterial blood pressure, and, possibly, lower oxygen consumption of tissues(2). It is therefore reasonable to assume that this drug might alter cholesterol metabolism and influence the usual responses of rabbits to cholesterol feeding. The present experiment was designed to study the effect of chlorpromazine in this connection.

Method. Twenty-four albino rabbits weighing from 1810 to 2320 g initially were used. Nine received daily, 10 mg/kg intramuscular injections of chlorpromazine (Thorazine hydrochloride; Smith, Kline and French Laboratories)† and were fed rabbit food pellets containing 1% cholesterol(3). Nine rabbits were given equivalent injections of chlorpromazine but were fed the stock diet without added cholesterol. Six rabbits received only the cholesterol diet. The animals were given a daily ration of 100 g and food consumption was recorded daily. Body weights were determined every other week. Blood (9-10 cc) was drawn by ear vein initially and at the end of the 1st, 3rd, 6th, and 9th weeks. Free and esterified cholesterol, phospholipid, total lipid, proteins and sugar were determined on the serum as described previously(3). Determinations of serum bilirubin (Evelyn and Malloy) were also attempted but the levels were always too low to obtain quantitative readings. All animals survived and were sacrificed at the end of the tenth week. Complete gross and microscopic studies of the viscera were performed. The aortas were stained grossly with Sudan IV and the distribution of lipid plaques charted as previously described(3).

Results. Blood chemistry: Cholesterol levels rose progressively in all cholesterol fed animals but by the end of the third week it was apparent that the chlorpromazine group had significantly lower values (Table I). At the ninth week the total cholesterol of this group was only about one-half as high (Mean 985, Max. 1368, Min. 760 mg/%) as that of those fed the 1% cholesterol diet only (Mean 1834, Max. 2166, Min. 1535 mg/%). The ratio of free to esterified cholesterol did not change significantly in either group. Total cholesterol rose slightly and transiently in 8 of 9 chlorpromazine rabbits on stock diet reaching its peak during the third week. In these 8 rabbits the mean cholesterol was 322 mg/% (Max. 457, Min. 234). No explanation can be given for the failure of the 9th rabbit to show this change. It fell in all 8 to a mean level of 157 mg/% by the sixth week and was within normal limits at the ninth week. The free and ester fraction ratios were not altered during this temporary rise.

* This investigation was supported by Research Grant from National Heart Institute of N. I. H., Public Health Service.

† We wish to thank the Smith, Kline & French Laboratories for donating this preparation.

TABLE I. Total Serum Cholesterol in Cholesterol Fed and Chlorpromazine Treated Rabbits.

	Total serum cholesterol, mean mg %				
	Wk of exp.				
	0	1	3	6	9
Chlorpromazine only	61	68	293	157	95
Chlorpromazine and cholesterol	55	411	759	961	985
Cholesterol only	65	374	1140	1560	1834

Serum phospholipids (Table II) were also elevated in all cholesterol fed animals but to a lesser degree than serum cholesterol. The ratio of cholesterol to phospholipid was very similar in both the chlorpromazine and non-chlorpromazine treated, cholesterol fed rabbits. It varied from about 3:1 to 2:1 in most determinations. Phospholipid values were also slightly elevated in the third and sixth weeks in rabbits receiving only chlorpromazine but generally lagged behind the slightly elevated serum cholesterol. There was, however, no significant change in the ratio of these 2 lipid fractions. Neutral fat (calculated as previously described(4)) was slightly elevated in all cholesterol fed animals especially in the latter weeks of the experiment, varying from 0.48 g % to 0.82 g %. There was no significant difference between the chlorpromazine-treated and non-chlorpromazine treated cholesterol fed groups. Neutral fat levels remained low and remarkably constant throughout the experiment in the group receiving only chlorpromazine. Serum globulin tended to rise in all chlorpromazine treated rabbits but this was inconstant in degree. A few had reversed albumin/globulin ratios on several occasions. Blood sugar and serum bilirubin were never significantly elevated.

Body weight and food consumption. All 24 rabbits consumed their entire daily 100 g food ration except on very few occasions when 10-20 g of food was left in the containers. None developed diarrhea. All animals gained weight consistently throughout the experiment. The mean weight gain in the group receiving both chlorpromazine and cholesterol was significantly less (58 g/wk) than in the groups receiving only chlorpromazine (103 g/wk) and cholesterol only (98 g/wk). All animals were in good health throughout the experiment and after the first 2 injections of chlorpromazine showed no obvious signs of sedation. Body temperature as measured intraperitoneally with a telethermometer during the eighth week of the experiment was not lowered significantly in chlorpromazine treated rabbits.

Necropsy findings. A striking inhibition of lipid deposition in the aortas, heart valves, and pulmonary arteries was noted in all but one of the cholesterol fed animals that received chlorpromazine (Fig. 1). In these 8 only a few thin patches of lipid were found chiefly in the aortic arch. One rabbit had fairly extensive plaques, but even in this animal the lesions were less extensive than in all but one of the group receiving cholesterol only. Such individual variation is a common finding in cholesterol feeding experiments in rabbits. Rabbits receiving chlorpromazine only had no gross vascular disease.

The livers of the rabbits receiving both chlorpromazine and cholesterol were smaller (mean wt 103 g) and less yellow than those that received cholesterol only (mean wt 125 g). They were only slightly larger and paler than the livers of animals that received only chlorpromazine (mean wt 95 g). Histologically, the liver cells of the chlorpromazine

TABLE II. Serum Phospholipid and Neutral Fat in Cholesterol Fed and Chlorpromazine Treated Rabbits.

Group		Wk of exp. 0	1	3	6	9
Chlorpromazine only	Phospholipid (mean mg %)	109	74	175	141	91
	Neutral fat (mean g %)	.35	.39	.45	.38	.42
Chlorpromazine and cholesterol	Phospholipid (mean mg %)	51	277	445	377	431
	Neutral fat (mean g %)	.38	.48	.60	.55	.55
Cholesterol only	Phospholipid (mean mg %)	67	210	496	600	771
	Neutral fat (mean g %)	.38	.43	.27	.61	.82

treated, cholesterol fed rabbits were less swollen and contained less finely dispersed lipid droplets than did those of the cholesterol fed animals that received no chlorpromazine. No evidence of liver cell damage or bile stasis was noted in any chlorpromazine treated rabbits. Enlargement and increased lipid storage was equally prominent in the adrenal cortices of both cholesterol fed groups. Histologic study of the other viscera revealed no significant changes that could be attributed to chlorpromazine administration. The mean heart weight of all 3 groups was approximately the same.

Discussion. It seems likely that the reduced amount of lipid deposited in the arterial walls in chlorpromazine treated, cholesterol fed rabbits is related to the lower levels of blood cholesterol observed in these animals as compared to the cholesterol fed rabbits that did not receive this drug. Except under special circumstances the degree of arterial lipid deposition in cholesterol fed rabbits varies directly with the degree of hypercholesterolemia in our own experience. There is no evidence that alterations in blood lipid interrelationships such as lowered cholesterol/phospholipid or cholesterol/neutral fat ratios played any role. It is also clear that malnutrition or general debilitation were not a factor in inhibiting arterial lipid deposition since the chlorpromazine treated animals remained in good health throughout the experiment. The degree of inhibition of lipid deposition was greater than that observed previously(3) in cortisone treated, cholesterol fed rabbits on a comparable regimen. If generalized depression of metabolism was involved such as occurs in hypothyroidism it might be expected that chlorpromazine would have accentuated hypercholesterolemia and enhanced arterial lipid deposition. This was obviously not the case. The failure of chlorpromazine treated, cholesterol fed rabbits to gain as much weight as the control groups, suggests the possibility that even though these animals consumed as much cholesterol, its absorption from the gut may have been reduced. However, the mean weight gain in the rabbits receiving only chlorpromazine was as great as in those fed cholesterol only.

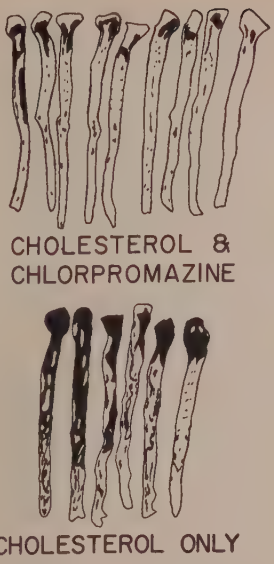

FIG. 1. Extent of aortic lipid deposition in rabbits fed cholesterol and inj. with chlorpromazine (upper row) compared with that in rabbits fed cholesterol only (lower row). Black areas represent portions of the intima covered by sudanophilic lipid deposits.

The inhibitory effect of chlorpromazine on hypercholesterolemia in these rabbits may have been mediated through the liver. Blood cholesterol levels are believed to be closely regulated by the metabolism of this lipid in the liver(5). To support this interpretation is the finding that the livers of the chlorpromazine treated animals failed to enlarge and to store as much lipid material as did those of rabbits that received only cholesterol. Chlorpromazine is known to produce jaundice occasionally and presumably some sort of liver injury in man although the exact mechanism has not been elucidated. The transient rise in serum lipids and globulin during the 3rd and 5th weeks in rabbits receiving only chlorpromazine was greater than ordinarily occurs in untreated rabbits and was therefore presumably due to the drug. These changes suggest that a brief period of metabolic readjustment may have occurred. This period corresponds somewhat to the time at which jaundice appears most frequently in man after chlorpromazine therapy is instituted. In some instances these attacks of jaundice in

man have been associated with moderate elevations of serum cholesterol(6). This has usually been explained on the basis of biliary stasis. There appears to be little evidence that chlorpromazine has any striking effect on the usual blood lipid levels found in man. Hollister and Kanter(7), however, report that it may be effective in lowering blood lipid levels of patients with essential hyperlipemia.

Summary. The blood cholesterol levels of cholesterol-fed rabbits given daily intramuscular injections of chlorpromazine fail to rise as high as in rabbits fed cholesterol only. Deposition of lipid in arteries and in the liver is also greatly reduced. Repeated injections of chlorpromazine in rabbits on stock diets may cause transient periods of mild hypercholesterolemia.

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Studies with 5-Bromouracil in Rodents and Dogs.* (22684)

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The growth inhibitory activity of 5-bromouracil was first observed in *Lactobacillus casei*(1). Later the substance was shown to be extensively incorporated in lieu of thymine in the deoxyribonucleic acids of bacteria(2, 3,4) and of a T_2 phage of *Escherichia coli*(4). Such findings suggest that the agent may be a thymine antagonist—a supposition supported by the near identity in size of the bromine constituent and of the methyl group of thymine (2). Anti-thymine actions may also be involved in the potentiation by 5-bromouracil of the inhibitory effects of folic acid antagonists in *L. casei*(5). Because of these findings, it has become of interest to ascertain whether 5-bromouracil might potentiate the action of amethopterin in the treatment of human leukemia.† With this in mind the work presented here was initiated as a preclinical study of the pharmacological actions of 5-bromouracil. During the study special interest arose in its catabolic fate *in vivo* because of the presence of crystalline deposits in kidneys or bladders of intoxicated animals. It is the primary purpose of this report to document this finding and to present information concerning the chemical nature of these deposits.

Procedure. Mice, rats and dogs were the subjects of the present investigation. The

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The 5-Bromouracil was purchased from the Dougherty Chemical Co., New York City.

† Personal communication from Dr. J. H. Burchenal.

TABLE I. Toxicity of 5-Bromouracil in Rodents.

Species	Administration	No. of successive daily doses	LD_{50}* (mg/kg/day)	19/20 confidence limits* (mg/kg/day)	S*
Mice	Intraper.	1	1400	1120–1750	1.25
	"	5	700	†	1.13
Rats	Intraper.	1	1700	1420–2040	1.16
	"	5	710	530– 960	1.42
	Oral	5	1410	1040–1900	1.42

* Calculated according to Litchfield and Wilcoxon(11). All animals were observed for 14 days after end of treatment.

† Confidence limits not calculated since mortality for doses used was either >84% or <16% (11).

mice in all cases were male Swiss albino mice (Millerton Research Farms). Male CFW (Carworth Farms Wistar) rats were employed for the toxicological studies while the pathological studies were carried out with the male CFN rats (Carworth Farms Nelson). The latter are a sub-strain of Wistar rat which has been bred free of "chronic murine pneumonia," salmonellosis and bartonellosis (6,7). They tolerate larger doses of most agents which we have recently tested than do CFW rats. Most of the toxicological, hematological and biochemical methods employed have been described (8,9). The 5-bromouracil was administered as suspensions in 0.9% NaCl containing 0.5% CMC (carboxymethylcellulose). The mice and rats received 1 ml of suspension per 100 g of body weight given either intraperitoneally or by stomach tube. The total daily oral dose for each dog was suspended in 50 ml of the saline CMC solution and administered by stomach tube using 50 ml of water to rinse the tube. For intravenous administration in dogs the agent was solubilized in 0.9% NaCl by the addition of molar equivalent amounts of ethanolamine. Because of limitations of solubility it was not practical to give intravenous doses greater than 200 mg/kg/day. Plasma and serum bromide levels were determined by the method of Brodie and Friedman(10). When 5-bromouracil was added to plasma or serum this method did not give rise to the liberation of bromide ion.

Results. Course of intoxication in rodents. Table I presents toxicity studies of 5-bromouracil in mice and rats. The toxicity of this agent was similar in both species. Single lethal doses, 2 g/kg, caused death within 24 hours. Following such doses the animals became weak, dyspneic, and depressed within 2-3 hours and the depression increased progressively until death. Several hours after the injections some animals had red tears and a few showed an orange urine. In animals treated with successive daily doses whether by the intraperitoneal or oral route, depression was noted several hours after each administration of the higher doses (1000 mg/kg/day intraperitoneally or 2000 mg/kg/day by mouth.) Deaths occurred between the second and seventh days in these groups. Ruffled fur was also prominent at 2 to 4 days in the animals given 500 to 1000 mg/kg/day and red-brown lacrymation as well as deep orange urine was observed. Two weeks after the last of these doses the survivors appeared healthy and the experiment was discontinued. At lower doses rodents showed no signs of intoxication. As will be seen below, the administration of 5-bromouracil gives rise to significant concentrations of bromide ion in plasma. This suggested a possible explanation for the depression noted in the treated animals. To test this possibility 2 groups of rats (6 rats/group) were given single, intraperitoneal injections of molar equivalent amounts of bromine as 5-bromouracil and NaBr, 2 g/kg and 1.08 g/kg, respectively. The 5-bromouracil animals died within 24 hours whereas the animals receiving sodium bromide recovered after a period of depression. At one-half of these doses both groups of 6 animals survived with no overt signs of intoxication. These results indicated that the liberation of bromide *in vivo* cannot be solely

TABLE II. Chromatographic and Spectral Findings.

	R_f	Maxima pH 5	Maxima pH 13	Minima pH 5	Minima pH 13
Uracil standard	.25	259	284	228	242
5-bromouracil standard	.37	274	289, 226	247	258
A. Rat kidney deposits	.40	275	290, 226	243	255
B. Rat urine spot 1	.26	259	283	228	242
2	.39	274	289, 225	244	256
C. Dog bladder deposits	.40	275	290, 226	242	256

responsible for the acute deaths caused by 5-bromouracil.

Pathological changes in rats. As mentioned above, pathological changes were studied in CFN rats. For this purpose, 18 rats were given 2 g/kg/day by oral intubation for 4 consecutive days. Six rats, chosen at random, were sacrificed at 96 hours (that is, at 24 hours after the last dose). At this time the average weight loss was 15%. The remaining animals, which had also lost weight, were observed for an additional 2 weeks; at the end of this period they had regained their weight and appeared healthy. In all the sacrificed rats the spleen appeared small. In 2 of the animals, congestion and hemorrhage were noted in the cervical lymph nodes. The kidneys of all were enlarged and, when transected, the cortices appeared edematous. Yellowish-gray crystalline deposits encrusting the papilla were found in the pelvis of each kidney. The bladders were filled with bright red urine. The principal microscopic changes were observed in the urinary tract. In all animals an internal hydronephrosis was present associated with small foci of acute pyelonephritis. Only rare tubular deposits were observed in the papillas. These were pink amorphous masses which only rarely contained needle-like structures. Since many crystalline masses were observed grossly, the paucity of deposits observed microscopically may be accounted for by loss during histological processing. More often rounded bodies which stained dark blue were seen in the medulla. These had the appearance of calcospherites. The basement membranes of all glomerular capillaries were slightly and diffusely thickened. Most of the convoluted tubules contained protein casts. A mild cystitis was present in the bladder associated with edema of the wall; edema was also observed in the prostate. In one animal pink masses similar to those observed in the papilla were present in the lumen of the bladder. In the spleens the red pulp appeared to contain fewer nucleated elements than in control rats. Sections of sternum revealed marked congestion of the marrow. In 5 animals there was a slight decrease of nucleated elements which varied from 10 to 20%. In 1 animal the decrease was 50%. The remaining tissues including lymph nodes, intestines and liver showed no significant changes. The kidney deposits from 2 of the rats mentioned above were removed with a spatula and saturated aqueous solutions were prepared. Descending paper chromatograms were developed with these solutions using Whatman No. 3 MM paper and n-butanol saturated with water. About 20 γ of material was applied to each spot at the origin. After development of the chromatograms, the ultraviolet absorbing components were visualized by means of a 15 watt General Electric Sterilamp equipped with a Corning No. 8963 filter. In this case, as in all chromatographic studies reported herein, uracil and 5-bromouracil‡ standards were used. The ultraviolet absorbing spots were eluted and read against appropriate blanks at pH 5 and pH 13. The results are shown in Table IIA. The R_f as well as the spectrum in water and alkali indicated that for the most part the crystalline material was unchanged 5-bromouracil.

Metabolic fate in rats. Additional studies were made of the fate of 5-bromouracil in rats (CFW). Six animals were placed in individual metabolism cages without food but with free access to 0.9% NaCl in drinking bottles

‡ The 5-bromouracil standard employed was the same as that used in the animal studies. It contained no discernible uracil (Table II).

and were starved for 18 hours prior to and during the experimental period. Three received intraperitoneally 0.5% CMC in saline and the other 3 1 g/kg of 5-bromouracil. The 24-hour urine from each rat was collected and diluted with 2 parts water. Three ml portions were then evaporated to 0.2 ml and the concentrated urines were chromatogrammed as described above. Little or no ultraviolet absorbing material was noted in the control urines. Two distinct spots (No. 1 and No. 2 —Table IIB) were observed on the chromatograms of the urines of rats receiving 1 g/kg. From the R_f of these spots and from their absorption spectra they corresponded to uracil and unchanged 5-bromouracil, respectively. No attempt was made to determine quantitatively the amounts nor ratio of the components present. It should be noted that plasma bromide levels at this time were 26-27 mg/100 ml in the treated animals as compared to 0 in the control rats. Since bromide ion is considered to be distributed throughout the extracellular fluid, that is, in about 25% of body weight, this plasma level would have resulted from the dehalogenation of about 12% of the 5-bromouracil injected. This probably represents the minimum amount of 5-bromouracil which was converted *in vivo* to uracil.

Course of intoxication in dogs. All animals were given a maximum of 10 successive daily doses excepting weekends and were observed for 2 weeks following the last dose. Intravenous doses of 100 and 200 mg/kg/day in 2 respective pairs were well tolerated and the animals showed no untoward signs. Two dogs which received 1 g/kg/day by mouth as well as 1 of 2 dogs which received 0.5 g/kg/day died during or shortly after treatment ended, that is, at 6, 14, and 16 days, respectively. Anorexia was noted at 2 to 6 days prior to death in these 3 dogs. Other signs of intoxication were weight loss and diarrhea. One of a pair of dogs receiving 250 mg/kg/day orally showed a transient anorexia and weight loss but both of this pair survived and were healthy at the termination of the experiment.

To determine the nature of lesions associated with intoxication, 2 dogs were given 1 g/kg/day orally for 4 consecutive days and sacrificed 24 hours after the last dose. At this time biochemical and hematological analyses revealed no significant changes from pretreatment values in the following: glucose, clotting time and bromosulfalein retention in blood; chloride, protein and non-protein nitrogen in plasma; hematocrit, total and differential counts of leucocytes and counts of reticulocytes in blood; and counts of nucleated cells in aspirates of bone marrow from the iliac crest. There was present, however, in the serum of both dogs a high concentration of bromide: 62 mg/100 ml. One of the pair presented a normal picture at autopsy. In the other dog the bladder was found to contain dark red urine which was positive for blood. The platelet count in the blood of this animal had decreased from the initial value of 140 x $10^3/mm^3$ to 7 x $10^3/mm^3$ at time of sacrifice. This dog's bladder was also filled with a dark tan solid mass. Its kidneys appeared normal.

The solid mass was removed from the bladder for further study and the findings are summarized in Table IIC. The chromatographic and spectral studies showed that the only detectable ultraviolet absorbing material present was 5-bromouracil.

In the same animal the only significant lesion observed microscopically was the presence of a few small mucosal hemorrhages in the wall of the bladder. Sternal and vertebral bone marrow, lymphoid tissues and intestine as well as all other tissues were normal. Neither crystals, hydronephrosis nor pyelonephritis were observed in the kidneys. In the other dog no microscopic changes were present in *any* of the tissues.

Discussion. It has been reported that 5-iodo-2-thiouracil is converted to urinary thiouracil in the dog(12) and is de-iodinated in the human(13). While this manuscript was in preparation Barrett and West(14) reported a study of a variety of halogenated pyrimidines in the rat. They found that parenteral administration of 5-bromouracil (40 mg/rat x 1) led to the urinary excretion of uracil which was identified by its behavior in several paper chromatographic systems.

In the present study, it was found that oral administration of 5-bromouracil (1 g/kg x 1)

to the rat gave rise to the excretion of both uracil and 5-bromouracil. The urinary components were studied by spectrophotometric as well as paper chromatographic technics. It was of interest to note a concomitant rise in plasma bromide levels. It was also of interest to note that when 5-bromouracil was given in high repetitive doses to rats and dogs, the foreign deposits which appeared in the kidney of the former and in the bladder of the latter were composed predominantly of unchanged 5-bromouracil. Although crystals were found in only one of the 2 dogs sacrificed, the serum bromide levels in both dogs were equally elevated. Presumably *in vivo* dehalogenation readily occurs in this species. The present investigation has revealed no clear-cut evidence regarding the cause of death in dogs given 5-bromouracil. It may be suggested that crystallization of the agent in the urinary tract and bromide poisoning may both contribute to intoxication. If this agent is to be considered feasible for clinical trial there is a real danger of the development of renal insufficiency or bromism or both as judged from the present findings which were consistent in both rats and dogs.

It should be pointed out that although microbiological evidence indicates that 5-bromouracil interferes with nucleic acid synthesis, lesions were not found in the bone marrow or intestinal epithelium of dogs given toxic doses. Only minimal changes in bone marrow were observed in rats given high doses of 5-bromouracil which were without discernible effect in the intestine of this species. This is in contrast to other agents, such as 6-mercaptopurine(8), which are believed to interfere with nucleic acid synthesis and which have primary sites of action in proliferating tissues such as the bone marrow and the intestine.

Summary. The toxicity of 5-bromouracil has been studied in mice, rats and dogs. Single lethal doses in rodents caused death within 12 to 24 hours. Somewhat smaller amounts when given repeatedly in daily doses gave rise to crystals in the kidney. These have been shown by chromatographic and spectrophotometric methods to be predominantly unchanged 5-bromouracil. The urines of rats given a single high dose of the compound have been found to contain uracil and 5-bromouracil. In dogs deaths are produced by the oral administration of 0.5 to 1.0 g/kg daily excepting weekends for a total of 10 doses. The principal changes noted in fatally intoxicated dogs were weight loss, anorexia, diarrhea and significant concentrations of bromide ion in plasma. In one such animal, the bladder with filled with a deposit of 5-bromouracil.

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Cystine Requirement for Normal Poliovirus Action on Monkey Kidney Tissue Cultures.* (22685)

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With the development of medium 199 Morgan, Morton, and Parker(1) provided a chemically defined medium which has been found to maintain monkey kidney cells in tissue culture during viral action. More recently, Eagle(2) studied the growth requirements of HeLa cells and mouse L cells and devised for these 2 cell types growth media which are chemically defined except for nondialysable serum components. At this laboratory Eagle's medium for HeLa cells without serum has been found to be adequate not only for the maintenance of monkey kidney cells but also for poliovirus multiplication in these cells. Thus a relatively simple chemically defined medium is available for the study of the relationship between cell nutrition and poliovirus action. The effect of the withdrawal of specific metabolites from this maintenance medium on poliovirus action is the subject of this paper.

Materials and methods. Monkey kidney cell cultures. Trypsinized cells were grown in medium D(3) for 6 days either in test tubes or in 60-mm diameter Petri plates. After removal of the growth medium, the established cultures were washed with phosphate-buffered saline (PBS)(4): the tubes 3 times, each time with 2 ml, and the plates 3 times, each time with 4.25 ml. These cells were then maintained either on Eagle's medium for HeLa cells without serum, here referred to as Eagle's maintenance (E_m), or on E_m minus one or more of its component compounds. *Poliovirus strains.* Plaque-purified pools of 3 strains have been used. These are Akron purified pool #1 (PPl), Brooks PPl, and Mabie PPla, which are examples of antigenic types I, II, and III, respectively. These strains have been described previously (5,6,7). When not in use, the pools are stored in a dry-ice box. *Nutritional tests in tube cultures.* To each tube culture of monkey kidney cells bathed by .90 ml E_m or deficient E_m at pH 7.2 to 7.3 is added .10 ml of a 1000-fold dilution in PBS of one of the poliovirus pools. This provides inocula of 740, 8500, and 300 plaque-producing particles (PPP) per tube for Akron PPl, Brooks PPl, and Mabie PPla, respectively. Control tubes receive .10 ml PBS. The tubes are rolled at 36°C and examined for cytopathogenic action on the second day after inoculation and daily thereafter usually through the eighth day after inoculation. *Nutritional tests using the plaque method.* The method of Dulbecco and Vogt(4) as adapted for use in this laboratory(7) has been used with the modification that the plaques develop under E_m or deficient E_m agar in an incubator continuously flushed with 97% air and 3% carbon dioxide(4). *Sources of compounds tested.* All compounds were purchased from commercial firms except thiomalic acid and 2-mercaptoethylamine hydrochloride of which free samples were kindly supplied by Evans Chemetics, Inc., New York City. None of the compounds was further purified.

Results. Delay in appearance of cytopathogenic effect on monkey kidney cells in tube cultures in absence of cystine. The cytopathogenic action of Akron, Brooks, and Mabie is delayed when cystine is not supplied for the monkey kidney cell-poliovirus system (Table I). Similar experiments have shown that the omission of all 8 vitamins or of any one of the amino acids tyrosine, leucine, methionine, arginine, histidine, lysine, isoleucine, phenylalanine, threonine, tryptophane, and valine normally present in E_m does not result in any delay in the cytopathogenic action of Akron PPl. The omission of glutamine has given variable results, sometimes resulting in a short delay and at other times in no delay.

Retardation of plaque development in absence of cystine. The result, obtained in tube

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Time of Appearance of Cytopathogenic Effect of 3 Strains of Poliovirus on Monkey Kidney Cells in Roller Tubes in Presence and in Absence of Exogenously Supplied L-cystine.

Poliovirus strain and pool*	Time required for completion of cytopathogenic action (mean day tubes positive)					
	1		2		3	
	Cystine†	No cystine‡	Cystine	No cystine	Cystine	No cystine
Akron PPl	3.0	≧6.8	4.2	≧7.8	3.0	≧8.0
Brooks PPl	2.9	≧7.6	3.2	≧7.5	3.0	≧7.2
Mabie PPla	2.9	≧7.2	4.0	≧7.8	3.0	≧7.8

* Each virus pool was diluted 1000-fold in PBS before inoculation.
† Medium E_m with L-cystine at .20 mM.
‡ " " without L-cystine.

cultures, that exogenously supplied cystine is necessary for the normal action of the Akron, Brooks, and Mabie viruses on monkey kidney cells has been confirmed using the plaque technic (Table II).

Delay in poliovirus propagation in absence of cystine. Curves which show the propagation of Akron PPl on monkey kidney tissue cultures in the presence and in the absence of cystine were obtained by plaque assays of daily harvests of supernatant fluids from appropriate cultures. These curves show that the propagation of Akron is delayed in the absence of cystine to about the same extent as is the cytopathogenic effect of Akron. This slower propagation in the absence of cystine probably is not due to any interference with virus adsorption (see below). It may be due either to a decreased rate of virus multiplication or virus release or both.

TABLE II. Plaques Formed by Akron PPl, Brooks PPl, and Mabie PPla on Monkey Kidney Cells in Presence and in Absence of Exogenously Supplied L-cystine.

Poliovirus strain and pool	Virus conc.	Plaques/plate	
		E_m*	E_m without L-cystine
Akron PPl	10^{-3}	—	1
	10^{-4}	†	0, 0
	10^{-5}	20, 25	0
	$10^{-5.4}$	11	—
Brooks PPl	10^{-4}	—	0
	10^{-5}	63, 28	0, 0
	10^{-6}	3, 4	0, 0
Mabie PPla	10^{-4}	46, 46	5, 1
	10^{-5}	8, 4	1

* Contains L-cystine at .05 mM.
† Too many to count.
— = Not done.
The plaques developed for 4 days at about 35°C. In addition to the differences in assay found above, the plaques that were formed on E_m without cystine were smaller than those formed on E_m.

TABLE III. Amount of L-cystine Necessary for Normal Cytopathogenic Action by Akron PPl on Monkey Kidney Cells.

Conc. (mM) of L-cystine in maintenance medium	Time required for completion of cytopathogenic action (mean day tubes positive)
0	≧9.1
.001	≧9.4
.004	≧9.9
.016	6.6
.064	4.3
.256	3.7

Each tube received .10 ml Akron PPl at 10^{-3} conc. in PBS.

Concentration of cystine necessary for optimal cytopathogenic action by Akron PPl. In Table III are presented data which show that the lowest concentration of L-cystine which will give optimal or near-optimal cytopathogenic action is about 10^{-4} M. The amount of cystine or "cystine-activity" in the inoculum itself has been estimated by the assay of autoclaved (10 lbs/in^2 for 10 minutes) samples of Akron PPl, Brooks PPl, and Mabie PPla, using the poliovirus-monkey kidney tissue culture system. In this way it was found that the amount of "cystine-activity" introduced into the system by the usual inoculum of 10^{-3} virus concentration is far too small to affect significantly the determination of the concentration of cystine necessary for optimal cytopathogenic effect.

Tests of compounds as possible substitutes for cystine. Seventeen compounds were selected on the basis of their metabolic relationship with cystine or simply because they possessed a chemical structure somewhat similar to that of cystine. These compounds were

TABLE IV. Activity of 17 Compounds in Replacing Cystine for Hastening Cytopathogenic Action of Akron PPl on Monkey Kidney Cells.

Activity relative to L-cystine		
Approximately equally active	Less active on a molar basis	Inactive‡
L-cysteine hydrochloride	DL-cystathionine*	DL-α-alanine
Glutathione	DL-homocysteine thiolactone hydrochloride†	Cysteic acid
		Glycine
		DL-homocystine (toxic; .1 mM)
		2-mercaptoacetic acid
		2-mercaptoethanol (toxic; .5 mM)
		2-mercaptoethylamine hydrochloride (toxic; .05 mM)
		DL-methionine§
		DL-serine
		Sodium sulfate
		" sulfite
		" thiosulfate
		Thiomalic acid

* About 8 times less active.
† " 20 " " " .
‡ Inactive at conc. of .10 and 1.0 mM except for the 3 compounds which were noticeably toxic for monkey kidney tissue cultures as designated above. Following the notation of toxicity is the highest relatively nontoxic conc. tested and found inactive.
§ Normally present in E_m at .05 mM.

tested for the ability to substitute for cystine in the latter's capacity to hasten the cytopathogenic action of Akron PPl. The results obtained permit the grouping of the compounds into 3 categories (Table IV). The possibility that the relatively low activities of DL-cystathionine and of DL-homocysteine thiolactone hydrochloride may be due to small amounts of contaminating substances, *e.g.*, cystine, has not been excluded.

Elimination of adsorption as the step at which cystine is effective. Particles of Akron PPl were adsorbed for 30 minutes at 36°C onto monolayers of monkey kidney cells, which had been washed 3 times with PBS (4.25 ml/washing), in the presence of 3 different media: PBS, E_m without cystine, and E_m with L-cystine at .50 mM. The fraction of the particles adsorbed was the same in the 3 media. This indicates that the presence of exogenously supplied cystine is effective at some postadsorptive phase(s) of the viral infection.

Discussion. Davies *et al.*(8) studied the effect of dietary amino acid deficiencies in mice on the course of poliomyelitis following intracerebral inoculation of a mouse-adapted strain of Lansing poliovirus. They found that a deficiency in any one of 8 amino acids (tryptophane, isoleucine, valine, phenylalanine, histidine, threonine, methionine, and leucine) resulted in some delay in the onset of symptoms, in a decrease in the total number of mice paralyzed by the 28th day postinoculation, and in an increase in the number of mice which died without showing characteristic signs of poliomyelitis. A lysine deficiency, however, had very little or no effect on the course of the disease. Their deficient mice and one of their control nondeficient groups received no cystine, cysteine, or glutathione. Their other control group received only the small amount of cystine present as residues in the casein fed. It is difficult to relate this work to the studies performed *in vitro* because of the manifold differences in system and technic. Recently Eagle and Habel have reported that salts, glucose, and glutamine are sufficient and necessary for poliovirus propagation on HeLa cells. A comparison of their findings with the results herein reported suggests that the cystine and possibly the glutamine requirements for poliovirus action depend on the nature of the host cell and/or the nature of the poliovirus strain. Further investigations, however, are needed to clarify the situation. Rappaport(9) has developed a medium containing salts, glucose, cysteine, and six other amino acids for the maintenance of monkey kidney tissue cultures during polio-

virus propagation. Furthermore, she has found that cysteine can replace the 6 other amino acids for poliovirus propagation. The combined evidence strongly indicates that cysteine and/or its disulfide derivative cystine play an important role in the process of poliovirus propagation in monkey kidney tissue cultures. These results suggest the possibility of finding cystine antimetabolites which would delay poliovirus action *in vitro* and perhaps *in vivo*. With only the information available at this time it is somewhat premature to speculate as to the reason for this cystine requirement. One reasonable working hypothesis is that the available endogenous cystine, either free or in peptide bondage, simply does not meet the demand for the cystine residues of the protein portions of the newly-forming poliovirus particles.

Summary. The cytopathogenic action of the poliovirus strains Akron, Brooks, and Mabie on either tube or Petri plate cultures of monkey kidney cells is considerably delayed when cystine is omitted from the maintenance medium. Omission of all the vitamins or of any one of the 12 other amino acids except glutamine normally present in the maintenance medium resulted in no delay in the appearance of the cytopathogenic effect of Akron PPl. Omission of glutamine resulted in a short delay or no delay. It has further been shown that the propagation of Akron is slower in the absence of cystine. The lowest concentration of L-cystine which will give optimal or near-optimal cytopathogenic action by Akron is about 10^{-4} M. Glutathione and L-cysteine hydrochloride can replace cystine at about the same concentration; DL-homocysteine thiolactone hydrochloride and DL-cystathionine can replace also but only at higher molar concentrations. None of 13 other compounds was found to replace cystine. The adsorption of Akron onto monkey kidney cells is not affected by the absence of cystine.

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Role of the Adrenal in Response of Rats to *Orinase*.* (22686)

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Adrenocortical and medullary hormones both counteract the hypoglycemic effects of insulin(1,2,3). Excessively high doses of the hypoglycemic agent *Orinase* do not produce a hypoglycemic response(4).† These observations suggested that the adrenals may respond to high doses of *Orinase* with increased secretions. Therefore, experiments were designed to study the role of the adrenal cortex and medulla in modifying the hypoglycemic reaction of rats to this drug.

Methods. Male rats obtained from the Upjohn colony (Sprague-Dawley ancestry) were used throughout these experiments. The intact animals weighed 140-160 g at the time of treatment with *Orinase*. Adrenalectomies and

* Upjohn tradename for 1-butyl-3β-tolylsulfonylurea.

† Personal communication from W. L. Miller.

adrenal demedullations were performed at an average body weight of 150 g. Adrenalectomized rats were maintained on 1% NaCl as drinking fluid and were given the hypoglycemic drug on the 7th day postoperatively except in one experiment. In this case, as shown in Table II, it was administered on the 9th day after adrenalectomy. The O day represents the day of *Orinase*. The 500 μg/day dose of hydrocortisone was started on the 4th day postoperatively and given for 5 days, with the last injection given immediately prior to the *Orinase*. The 5 mg dose of hydrocortisone was given as one injection immediately prior to the sulfonylurea on day O. All hydrocortisone injections were given in a volume of 0.2 cc of CMC vehicle(5). When epinephrine‡ was used it was given subcutaneously 1 hour following the oral administration of the hypoglycemic agent. The adrenal demedullated rats were kept at least 4 weeks before *Orinase* treatment in order to insure regeneration of the adrenal cortices. The functional activity of the adrenal cortices has been indicated§ by the fact that these animals can withstand stress induced by intraperitoneal egg albumin and the glands weighed between 40-50 mg after 4 weeks. These rats were maintained on 1% sodium chloride as drinking fluid for the first week and then on tap water. *Orinase* was given orally in all cases. Doses up to 275 mg/kg were given in 0.5 cc of CMC vehicle(5) and higher doses in a volume of 2 cc. All animals were fasted 24 hours prior to *Orinase* administration. Blood samples were obtained from the vena cava 2½-3 hours after *Orinase* treatment while the animals were under sodium cyclopal anesthesia. The blood was oxalated and glucose was estimated by the Folin-Wu method(6). Liver glycogen was determined by the anthrone method on tissues removed 7 hours following treatment.

Results. The minimal effective hypoglycemic dose of *Orinase* in intact rats was approximately 100 mg/kg (Fig. 1). These data also show that the maximal fall in blood sugars was 36% at a dosage of 266 mg/kg.

‡ Upjohn Epinephrine Hydrochloride 1:1000.

§ Unpublished data.

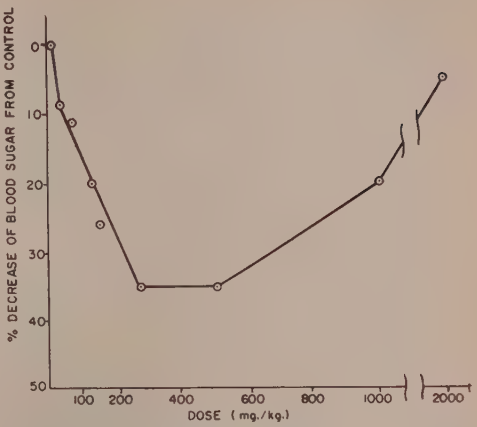

FIG. 1. Dose-response of intact rats to the hypoglycemic effects of *Orinase*. Each point represents 8 controls and 8 treated animals. Animals were bled 2½-3 hr following treatment.

At the high dose of 2 g/kg there was no demonstrable depression of blood sugar in the intact rats. The same type of response was manifested in the deposition of liver glycogen under the influence of *Orinase* (Table I). While 266 mg/kg of the sulfonylurea produced a marked increase in liver glycogen, the higher doses caused less deposition.

In adrenalectomized rats as little as 9 mg/kg of *Orinase* caused a fall in blood sugar (Fig. 2). At dosages of 45 and 90 mg/kg the adrenalectomized rats were subject to convulsions and blood sugar levels were depressed by 45 and 57% from the controls. To establish that the convulsions were due to hypoglycemia an experiment was performed in which *Orinase* at a dosage of 100 mg/kg was administered orally to 20 adrenalectomized rats. One-half of these animals received 1 g of glucose orally after they were in

TABLE I. Effects of Varying Doses of *Orinase* on Fasting Liver* Glycogen.

Treatment	Dose	No.	Liver glycogen
Vehicle	2 cc	7	.24
Orinase	266 mg/kg	6	.66
	523	6	.57
	1048	6	.35
	2000	6	.34

* Livers were removed 7 hr following treatment.

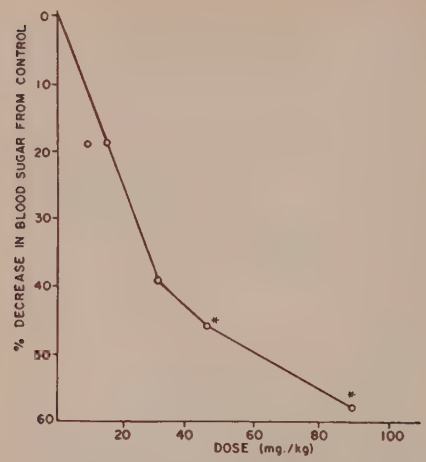

FIG. 2. Dose-response of adrenalectomized rats to *Orinase*. Each point represents 8 controls and 8 treated rats. Animals were bled 2½-3 hr following treatment.

* All animals were convulsing.

convulsions. In every instance the glucose stopped the convulsions and the animals were on their feet within 10 minutes. If glucose was not given, convulsions continued for several hours and usually ended in death.

In adrenal demedullated animals the minimal dose of *Orinase* causing hypoglycemia was between 15 and 30 mg/kg (Fig. 3). This is compared with 9-15 mg/kg for the hypoglycemic dose in adrenalectomized rats and 100 mg/kg for intact rats. Therefore, the adrenal demedullated animals are considerably more sensitive to the hypoglycemic effects of *Orinase* than the intact rat but do not seem to be quite as sensitive as the adrenalectomized animals. In addition, the dose of 2 g/kg produced essentially as much depression of blood sugar as the lower doses in the adrenal demedullated animals. It is important to note that the 2 g/kg dose was the maximum dose in this animal preparation since it killed approximately ½ of them.

To delineate further the role of the adrenal cortex and medulla in the response to *Orinase*, experiments were carried out to study the effects of hydrocortisone and epinephrine on blood sugar response of adrenalectomized rats to this drug. Treatment with hydrocortisone at 0.5 mg per rat per day for 5 days

TABLE II. Effects of Hydrocortisone on Response* of Adrenalectomized Rats† to *Orinase*.

No. rats	Treatment	Days	Dose	Blood sugar (mg %)
4	Subq. saline	5	.2 cc	91
	Oral CMC	0‡		
4	Subq. saline	5	.2 cc	
	Subq. F	0	5.0 mg	
	Oral CMC	0	.5 cc	103
4	Subq. F	5	.5 mg	
	Oral CMC	0	.5 cc	100
4	Subq. F	5	.5 mg	
	Oral *Orinase*	0	66.0 mg/kg	58
5	Subq. saline	5	.2 cc	
	Subq. F	0	5.0 mg	
	Oral *Orinase*	0	66.0 mg/kg	53§
5	Subq. saline	5	.2 cc	
	Oral *Orinase*	0	66.0 mg/kg	38§

* Blood was taken 2½-3 hr following *Orinase* treatment.

† Avg body wt at time of *Orinase* was 160 g.

‡ 0 day was 9th day after adrenalectomy and at end of indicated treatment. The F 0-day and last inj. of F 5 days was given immediately prior to the *Orinase*.

§ Convulsing.

prior to *Orinase* administration in the adrenalectomized rats partially reversed the hypoglycemic effects of *Orinase* and increased the blood sugar somewhat over that of the controls (Table II). It is also interesting that a

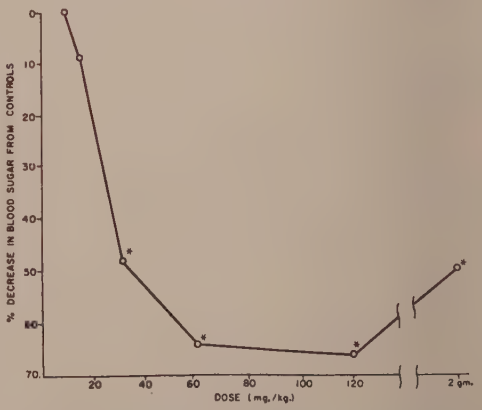

FIG. 3. Dose-response of adrenal demedullated rats to *Orinase*. Each point represents 8 controls and 8 treated animals except at the 2 g/kg dose. In this group there were 15 controls and 8 *Orinase*-treated as 7 treated rats died before they were bled. Animals were bled 2½-3 hr following treatment.

* All animals were convulsing.

TABLE III. Effect of *Orinase* plus Epinephrine on Blood* Sugar in Adrenalectomized Rats.

No. rats	Treatment	Dose	Blood sugar
5	CMC	0.5 cc	76
5	*Orinase*	66 mg/kg	40
7	*Orinase* +Epinephrine	66 " 200 μg/kg	116

* Blood was taken 2½-3 hr following the *Orinase*. Epinephrine was given 1 hr after the hypoglycemic drug.

single dose of 5 mg of hydrocortisone per rat given simultaneously with *Orinase* also partially reversed the extreme hypoglycemic effects with *Orinase* in this animal preparation. The blood sugar levels of the animals which received 5.0 mg of hydrocortisone were also somewhat higher than the untreated control adrenalectomized animals. The effect of epinephrine in reversing the hypoglycemic effect of *Orinase*, however, was more striking than that seen with hydrocortisone (Table III). When 200 μg/kg of epinephrine was administered to adrenalectomized rats 1 hour after oral administration of *Orinase*, the hypoglycemia was completely reversed and the blood sugar levels rose significantly above those of the untreated adrenalectomized controls.

Discussion. The difference in sensitivity of intact and adrenalectomized rats to *Orinase* shows that the adrenal is involved in the response of rats to this drug and agrees with previous observations(7) that adrenalectomized rats were more sensitive to the hypoglycemic effects of other sulfonamides than intact animals. The failure of high doses of *Orinase* to induce hypoglycemia or liver glycogen deposition in the intact rat may be the result of stimulation of epinephrine secretion by the adrenal medulla since epinephrine is known to increase blood sugar and deplete liver glycogen. The observations reported here which support this explanation are as follows: (1) the adrenal demedullated animals are more sensitive to the hypoglycemic effects of *Orinase* than the intact rats. (2) The hypoglycemic activity of 2 g/kg of *Orinase* was nearly as marked as that produced by lower doses in the demedullated rats. (3) Epinephrine completely reversed the hypoglycemic response of adrenalectomized rats to the sulfonylurea.

Since hydrocortisone partially reversed the hypoglycemic response of the adrenalectomized animal to *Orinase* and since adrenalectomized animals seem to be more sensitive to it than demedullated animals, it is probable that the adrenal cortex is also involved, but to a lesser degree. Another observation which suggested that the adrenal cortex may be stimulated with the high doses of *Orinase* was that there was slightly less hypoglycemic response at the 2 g/kg dose level as compared to that obtained with lower doses in adrenal demedullated rats.

These results also indicate that when intact animals are used to test hypoglycemic agents a wide range of dosages should be employed to be sure that the phenomenon of reversal of effects does not occur. Adrenalectomized rats are better test animals since they are extremely sensitive to hypoglycemic drugs and also the likelihood of masking the hypoglycemic effect by adrenal secretory activity induced by high doses is eliminated.

Summary. In the intact rat low doses of *Orinase* produced a significant depression of blood sugar and deposition of liver glycogen while higher doses were ineffective. The adrenalectomized rat was found to be approximately 10 times as sensitive and the adrenal demedullated rat 3-6 times more sensitive to *Orinase* than the intact rat. Hydrocortisone partially and epinephrine completely counteracted the hypoglycemic effects of *Orinase*. Failure of high doses of this sulfonylurea to produce hypoglycemia and liver glycogen deposition in the intact rats may be due to a stimulation of epinephrine secretion by the adrenal medulla. It is also suggested that glucocorticoid secretion is also stimulated by high doses of *Orinase* in the intact and adrenal demedullated animals.

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Tubular Reabsorption of Protein in Experimentally Produced Proteinuria in Rats. (22687)

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It has long been recognized that cells lining the proximal convoluted tubule of the kidney of various animal species are capable of absorbing colloid substances from the tubule lumen(1-4); the process has been named *athrocytosis*. Dock(5) and Addis(6) were the first to suggest that under *normal* circumstances plasma proteins are to some extent filtered through the glomerulus of the kidney and reabsorbed by cells of the tubules. Because of the enormous volume of glomerular filtrate, a concentration of protein in the glomerular filtrate of only 15 mg/100 ml would result in excretion in the human of about 27 g of protein in 24 hours if the protein were not reabsorbed by the tubule cells. The work of many investigators has contributed to the concept of glomerular filtration and tubular reabsorption of plasma proteins in normal and abnormal conditions. These reports have recently been reviewed by Rather(7). Since this review, the quantitative aspects of tubular reabsorption of protein in the normal rat has been studied by Sellers, *et al.*(8) and the mechanism of tubular handling of reabsorbed protein has been investigated by Oliver and co-workers(9). Theoretically, a decrease in tubular reabsorption of plasma proteins filtered through normal glomerular basement membrane could account for the protein found in urine in most instances of proteinuria. Actually, however, in glomerulonephritis there is evidence of glomerular damage, and even in cases of idiopathic nephrotic syndrome, where only basement membrane thickening is found to suggest changes in glomerular permeability, calculations based on clearance studies in patients with massive proteinuria lead to the conclusion that glomerular permeability to plasma proteins is increased (10). This still leaves open the question of whether proteinuria occurs solely because filtration through abnormally permeable glomeruli exceeds the normal ability of tubules to reabsorb protein, or whether a decrease in tubular reabsorption of protein is an added factor in production of proteinuria.

The dye T-1824 (Evans Blue) binds firmly to serum proteins and has been used to measure renal clearance of albumin in humans(11) as well as tubular reabsorption of protein in rats(5,8,12). The amount of dye accumulating in droplet form in cells of the proximal convoluted tubule is assumed to be a measure of reabsorption from the tubule lumen of dye-labeled serum proteins. In the present report, dye-labeling of serum proteins has been used to study the role of tubular reabsorption of protein in 2 types of experimentally produced proteinuria.

Materials and methods. Female Osborne-Mendel rats of about 150 g body weight were used. Proteinuria due to uranium administration was produced by intravenous administration of 0.174 mg uranyl acetate containing 0.1 mg uranium, in 1.0 ml of 0.15 M saline. Proteinuria due to anti-kidney serum was induced by a single intravenous injection of rabbit anti-rat kidney serum. The nephrotoxic serum was obtained from rabbits immunized by repeated intraperitoneal injections of glomerular fraction of perfused rat kidney homogenate. To obtain urine for pro-

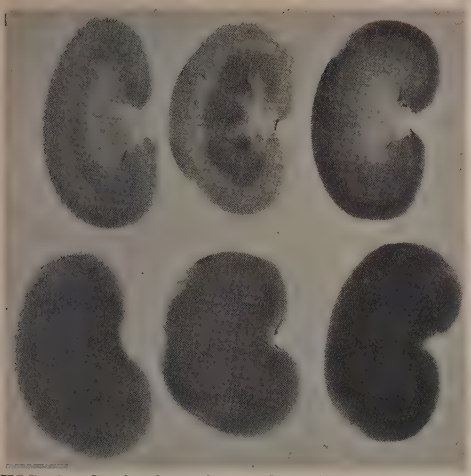

FIG. 1. Sagittal section and surface view of unfixed kidneys from control rat (left), rat with proteinuria produced by uranium inj. (center), and rat with proteinuria produced by inj. of nephrotoxic serum (right). Each rat received 2.5 mg T-1824 intrav. 24 hr before kidneys were removed.

tein determinations, rats were placed in individual metabolism cages with an adequate liquid diet containing casein hydrolysate in place of protein and urine was collected overnight. Proteinuria was measured by the method of Shevsky and Stafford(13). Two and one-half mg of T-1824 dye in 0.15 M saline were injected intravenously into a foot vein of rats followed by an overnight urine collection. Twenty-four hours after dye injection, the rats were anesthetized with ether, exsanguinated from the abdominal aorta, and both kidneys were decapsulated and removed. One kidney was sliced in half with a razor blade, placed in a Petri dish containing ice cold 0.15 M saline and examined in the gross and at various magnifications under a Leitz binocular dissecting microscope. The other kidney was fixed in 10% formalin and both frozen sections and hematoxylin and eosin and PAS stained sections were made. In another experiment, some animals were autopsied at daily intervals after antiserum injection and the kidneys were examined and weighed.

Results. Normal rats. After intravenous injection of 2.5 mg of T-1824 in normal rats, a normal straw or amber colored urine was obtained. Study under dissecting microscope of kidneys removed 24 hours after dye injection showed a pale green-tan or blue-green surface, and at higher magnifications none of the mosaic pattern of convoluted tubules was strikingly stained by the dye. On cut section (Fig. 1), the outer zone of the cortex was a faint but definite green, the inner zone was colorless and the medula was red. It was evident that relatively small amounts of the blue dye were present in the tubular cells.

Uranium proteinuria. It was observed in preliminary experiments, data from one of which are shown in Table I, that proteinuria was produced after a latent period of 2 or 3 days by 0.174 mg of uranyl acetate. This dose was chosen for 4 T-1824 experiments in 8 rats each. Forty-eight hours following injection of uranyl acetate, T-1824 dye was injected. Urine collected overnight was blue and contained protein. Twenty-four hours after T-1824 injection, the external surface of kidneys were a pale green color not unlike the T-1824 injected normal rat kidneys (Fig. 1), but at higher magnification the mosaic of tubules consisted of pale green colored tubules alternating with completely colorless tubules not observed in control rats. The cut section of kidneys showed a pale green outer cortex, containing somewhat less blue color than the T-1824 injected control kidneys, a colorless inner zone of cortex and the red medullary zone. Microscopic examination showed apparently normal glomeruli and changes in cells of proximal convoluted tubules consisting of marked necrosis and even desquamation of cells from the tubular basement membranes.

Nephrotoxic serum proteinuria. After intravenous injection of a standard dose of rabbit anti-rat kidney serum (twice the minimum amount required to produce proteinuria), the 150 g female rats developed immediate proteinuria. In most rats proteinuria persisted indefinitely, but in some rats it gradually subsided. Definite renal enlargement occurred in 2 or 3 days. T-1824 studies were made as early as 24 hours and at various longer periods after antiserum injection in 17 rats with chronic proteinuria due to nephrotoxic serum. The urine collected after T-1824 injection contained about 7 mg/hr of protein and was blue. When examined 24 hours after T-1824

TABLE I. Time of Onset of the Proteinuria Produced by Intravenous Injection of Uranium and Nephrotoxic Serum.

Material inj.	No. rats	Proteinuria Day 1	Day 2	Day 3	Day 4	Day 7
		mg/hr				
Saline (controls)	4	.3	.2	.1	.2	.2
.0178 mg uranyl acetate	2	.2	.1	.3	.3	.2
.178 mg " "	8	.3	.7	1.6	1.0	.5
1.78 mg " "	4	1.1	3.5*			
Nephrotoxic serum	4	11.0	5.1	4.0*		

* Sacrificed for autopsy examination.

injection the surfaces of kidneys were deep blue and under higher magnification one could see against the background of blue-stained tubules an occasional convoluted tubule which was an even more intense blue (Fig. 1). At highest magnification the blue could be seen to be in granular form, apparently in the tubule cells. The cut sections showed intense blue staining of outer and inner zones of the cortex; the medulla was red. Frozen sections of such kidneys after formalin fixation showed the blue granules or droplets in the cytoplasm of the cells lining the proximal convoluted tubules as has been described by other investigators in kidneys of normal rats 24 hours after intravenous injection of much larger amounts (25 mg) of T-1824(8,12). Varying degrees of basement membrane thickening and patchy lipid deposition in tubule cells were present.

It should be noted that the amount of T-1824 injected in our experiments was sufficiently small that it should have been completely bound to albumin even in chronically proteinuric rats with lowered plasma albumin levels. To avoid complication of the difference in plasma albumin levels between control and nephrotic rats, 2.5 mg of T-1824 were administered to 12 normal rats and followed within a few minutes in 6 rats by nephrotoxic antiserum. In those rats receiving nephrotoxic serum, there was deep blue staining of the cortex in 24 hours as described in the chronically proteinuric rats.

Discussion. Previous investigators have summarized the facts and the reasoning behind the assumption that the appearance of the T-1824 dye in the cytoplasm of proximal convoluted tubule cells represents dye-labeled protein which has been filtered through the glomerulus and reabsorbed from the tubule lumen by proximal convoluted tubule cells(5, 8,12). The T-1824 in serum and urine of proteinuric rats was all precipitated with the protein by protein precipitating agents in the experiments of Gilson(12), and Sellers, *et al.* (14), and in our own experiments.

In the present experiments, the injection of one-tenth of the amounts of T-1824 used in experiments of others with normal rats(12, 14) resulted in appearance of only a small amount of color in the cortex of normal rats after 24 hours. In rats with proteinuria resulting from anti-kidney serum administration, appearance of large amounts of T-1824 in the tubule cells probably indicates that dye-labeled protein entered the cells in larger amounts than in the T-1824 injected control rats. This is consistent with the hypothesis that an increase in glomerular permeability due to antiserum damage led to increased filtration of dye-labeled serum proteins through the glomerulus, followed by increased tubular reabsorption of dye-labeled protein. The greater than normal accumulation of blue dye in 24 hours in the tubule cells of rats which received T-1824 prior to nephrotoxic serum administration seems to us to eliminate the possibility that the accumulation of dye in nephrotic rats is due to the presence of a larger amount of unbound dye in nephrotic rats than in controls. An alternative explanation for our finding is that damage to the tubule cells interfered with the mechanisms for disposing of the reabsorbed dye so that an increase in the dye in tubules occurred without an increase in tubular reabsorption of protein. Consideration of the findings in the kidneys of rats with uranium damaged tubules makes this possibility seem unlikely.

The renal enlargement which occurs in rats receiving anti-kidney serum presumably is related to the increased reabsorption and perhaps degradation of filtered protein; this is suggested by the observation of similar enlargement when proteinuria was produced by repeated infusions of protein in normal rats (15).

The rats injected with 0.1 mg uranium intravenously developed proteinuria only after 48 to 72 hours. Uranium is transported in combination with serum albumin and bicarbonate(16). Since almost all the injected uranium leaves the blood stream, and concentration in the kidney is greatest in the first few hours(17), it is difficult to account for the delay in onset of proteinuria. Tubular reabsorption of uranium from the tubule lumen would account for the well known localization of uranium produced renal damage in the proximal convoluted tubule(17). It is also consistent with the concept that the proteinuria produced in uranium injected rats is due primarily to decreased tubular reabsorption of protein by damaged tubule cells since no increase, and in fact a probable decrease, in T-1824 accumulation by tubule cells is found. Since no glomerular damage is detected in the presence of obvious localized tubular necrosis in such rats, it is tempting to consider that the protein appearing in urine is normally filtered plasma protein which is no longer reabsorbed by damaged tubule cells. One cannot, however, rule out the possibility that an increase in glomerular permeability has occurred in addition to decreased tubular reabsorption of protein.

The findings indicative of increased reabsorption of dye-labeled protein by the tubule cells of the kidneys of rats injected with anti-rat kidney serum are in accord with similar observations made by Spector(18) using radioactive iodine as the tag for serum protein. It should be noted that the rats receiving antikidney serum develop hypoproteinemia and lipemia and have renal lesions which are similar to those observed in many cases of the nephrotic syndrome in man(19).

Summary. An attempt was made by labeling plasma proteins with the blue dye T-1824 to estimate the amount of protein filtered and reabsorbed by the kidney under several conditions. A single intravenous injection of 2.5 mg of T-1824 into normal rats did not cause the urine to be blue and the kidneys examined 24 hours after the injection exhibited only faint blue staining of the proximal convoluted tubules of the cortex. When proteinuria was produced by a single small injection of uranyl acetate, the injection of T-1824 was followed by blue staining of the urine. However, the cortices of the kidneys contained less blue dye than those of control animals, indicating, presumably, that there was less protein reabsorption than normal by the damaged tubular cells. Injection of the blue dye into nephrotic rats with proteinuria due to administration of nephrotoxic anti-rat kidney serum was followed by blue staining of the urine and intense blue staining of the cortices of the kidneys. This presumably indicated that protein reabsorption by the tubular cells was greater than normal, and that the proteinuria was a result of increased glomerular permeability and not of decreased tubular reabsorption of normally filtered protein.

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Genic Control and Hormonal Reversal of Sex Differentiation in Xenopus.* (22688)

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It has long been realized that in the cytodifferentiation of germ cells not only genes and external environmental factors play important roles, but also certain conditions of their immediate milieu(1). The first figure presents diagrammatically the basic facts and their interpretation based on recent work. Germ cells, on arriving in the primitive gonadal cortex, become invested each by a *nurse or follicle cell.* If unopposed, the follicle eventually induces development of the germ cell into an egg. However, if at the critical stage of sex differentiation a germ cell becomes located in the medulla, another inductor system, the *interstitial cells,* upsets the influence of the follicle cells and leads to the spermatogenetic type of differentiation.

How can such an interpretation be integrated with the well known facts of genic control of sex ratios? The present experiments seem to shed some new light on this problem.

Material and methods. *Xenopus laevis* Daudin has been bred in our laboratory since 1949 without addition of new stock. The experimental larvae were placed in hormone solutions of given concentrations, the solutions being completely replaced every 24 hours. The temperature was kept constant at 20°C.

Experiments. Since in the adult organism follicles and interstitial cells produce estrogenic and androgenic steroids, it was necessary to investigate the possibility that in embryonic sex differentiation the same hormones might first play the role of inductor substances. Experiments with Xenopus gave very interesting results, although not in the sense of a confirmation of the tentative assumption. Small or large doses of androgens added to the aquarium water equally produce what first seemed to be *all premature male cultures.* Given throughout the larval period they yield at metamorphosis little toads that all have well developed dark arm and hand pads and immediately start embracing each other. However, on dissection it is found that only about half of the animals have testes, the other half have normal ovaries (Fig. 2). We shall later come back to this experiment but next must describe the effect of the opposite treatment: exposing the larvae to estrogenic hormones, usually *estradiol.* Here the entire offspring is completely feminized. The original experiments with Allison(2,3) have often been repeated and thousands of offspring have been produced that consisted of females only(4,5). Some recent data are presented in Fig. 3.

These two hormone effects are all the more remarkable, because they are in contrast with the results of parabiosis and gonad transplantation, as described by Chang(6). The simultaneous presence of testes and ovaries in a system with a common blood circulation always results in normal testes and greatly suppressed ovaries. Sometimes even testicu-

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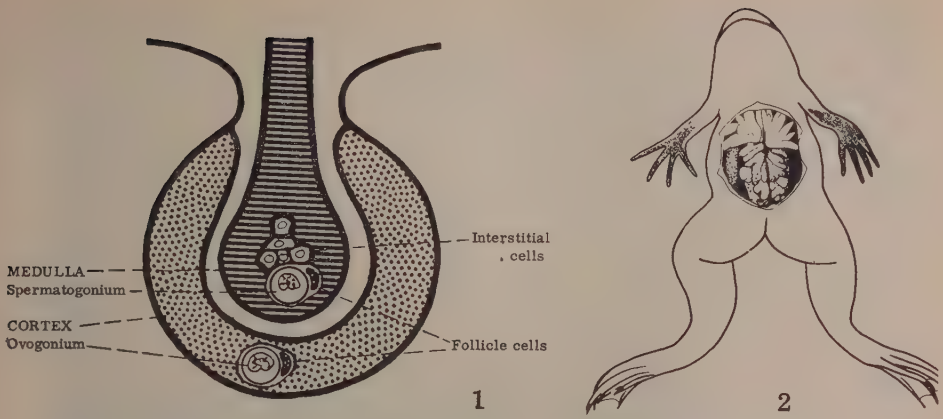

FIG. 1. Diagram of cortical and medullary inductors of sex differentiation.
FIG. 2. Genetic female (ZW) which while under testosterone administrations developed ovaries and male secondary sex characters.

lar nodules appear in the latter.

The striking difference between these reactions proves that the inductor substances are not steroid hormones; for even if the male parabiont were producing an androgen, our first experiment shows that it could only stimulate secondary sex characters but not inhibit ovarial development. On the other hand, if the female partner were liberating small amounts of estrogens, the genetically male co-twin should develop ovaries—which again is not so. What then is the nature *of the inductor substance?* Further analysis of this problem must be preceded by a clarification of the role played by the *sex genes* (Fig. 3).

In Xenopus normally the female is heterozygous (ZW) and the male homozygous (ZZ). Therefore, treating part of the lay of a normal pair with estradiol should give an all female offspring in which half are of ZZ constitution. This had to be verified in breeding tests, for both ZZ and ZW animals are of the same appearance. Of 43 tested in this way, 22 gave an F_1 generation with the normal 1:1 ratio (ZW females): but 21 produced entirely male offspring and thereby proved to be of ZZ constitution. Evidently we can now dispense with the heterozygous sex type. Breeding homozygous stock only, one may produce any desired number of females by estradiol treatments at the larval stage. The possibility of controlling the sex ratio at the will of the experimenter is often a great convenience.

These breeding experiments with sex reversed toads spread some revealing light on the problem of the nature of sex determining genes. Since estradiol need only be administered during a short larval period, it is evident that the entire process of ovogenesis and the development of female secondary sex characters, including sex behavior, are no longer under the control of the sex genes. Apparently the genes play an active role only during a short and definite period, starting a chain reaction which later runs off independently. In trying to locate this period we first treated several 100% ZZ (male) cultures for 1-week periods with estradiol, and soon found that the critical time lies at the transition of stages 26 and 27(7), which actually is the time of initial embryonic differentiation (Table I). Attempts to pinpoint more exactly the time of interference with gene action led to surprisingly clear results. The primitive undifferentiated gonad of Xenopus consists of a series of about 15 pseudosegments or gonomeres. In accordance with general vertebrate development, they form and mature in craniocaudal sequence. If 2-day administrations of estradiol are given at the end of stage 26, the tops of the gonads differentiate into ovary, but the lower part remains unaffected and develops according to the testicular type (Fig. 4b, 5b). If, however, treatment is delayed until the

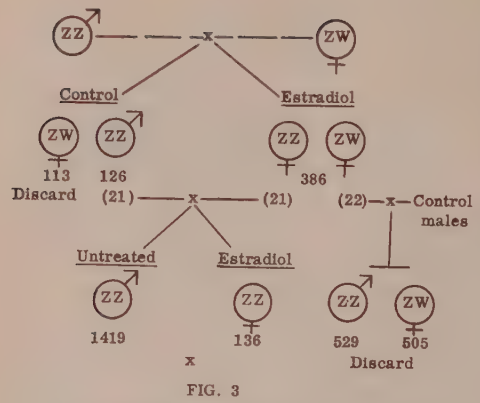

FIG. 3

FIG. 3. Experimental breeding record of Xenopus.

start of stage 27, the lower gonomeres become ovarial lobes. One may even produce triple sequences with ovary in the center and testis at both poles (Fig. 4a, 5a). In average cultures complete feminization requires close to 3 days. This means that about every 5 hours a new gonomere enters the responsive phase.

These experiments help in clarifying some aspects of the problem of *gene action*, recently discussed by Curt Stern(8). In general, the breeding of sex-reversed males in successive generations gives proof that the upset of genic expression neither induces, nor depends upon perceptible readjustments in the genic pool. This conclusion is unavoidable here, because the very cells that develop into eggs instead of sperms carry the genes into the subsequent generations. Also it becomes evident that the results of gene action are in time and quality co-determined by preadjusted factors of the internal milieu. In the present case the internal milieu is represented by inductor cells and substances. These are mostly proteins while the genes are DNA compounds. Delbrück has presented some interesting thoughts regarding the possible relationships between these widely different chemical classes(9). For a more penetrating discussion much more precise knowledge is needed especially regarding the inductor substances. In sex differentiation of gonochorists (*i.e.*, species with separate male and female sexes) there are at least two classes separately recognizable. Cortex and medulla each produces an agent, *corticin*

TABLE I. Series of All-Male (ZZ) Larvae, Each Treated during One of 4 Successive Weeks, with Estradiol, 50 μg/l.

Treatment	Stage (7)	N1*	N2†	♂	♀	♀
1st wk	24–25	47	38	38		
2nd	25–26	47	41	38	3	
3rd	26–27	72	65		4	61
4th	27–28	47	41	41		
Continuous	24–29	80	69			69
None		292	278	278		

* Original No. of tadpoles.
† No. of tadpoles survived.

and *medullarin* respectively, which induce ovogenesis or spermatogenesis. While in primitive hermaphrodite species the inductions by these substances may proceed side by side, even within one and the same gonad, in gonochorists the agents assume the character of antigens, so that the cortex now produces also an *anti-medullarin* and the medulla an *anti-corticin*. These "antibodies" have a wider range of action than the primary inductor substances and, as parabiosis experiments prove, they are proteins of high specificity that often are carried by the blood stream. These conclusions are partly a reinterpretation of previously reported experiments, but they are also based on the following new observations on Xenopus.

It was mentioned above that in male-female parabiosis, the ovary becomes extremely reduced. The same is the case if a testis is implanted into the body cavity of an early female larva. Interestingly, the same inhibition is not exerted by the testicular lobes of partially feminized males. Quite to the contrary, the ovarial lobes grow at about the same rate as ovaries of completely feminized larvae (Fig. 6). The full spermatogenic development in the testicular segment remains unaffected by the proximity of ovarial, also normally maturing lobes (Fig. 7). This is new evidence of the separate existence of the inductor of male differentiation (medullarin) and the inhibitor of ovarial differentiation (anti-corticin). Estradiol administration blocks the former only during actual treatment but the latter more generally and lastingly.

The rule that androgens have no sex-reversing effect on female larvae becomes

FIG. 4. Newly metamorphosed hermaphrodites of ZZ stock treated for 3 days with estradiol; a. ovarial gonomeres in the center of the sex glands; b. ovarial gonomeres above the (broader) testicular parts; × 19.

FIG. 5. Frontal sections through one sex gland each of 4a and 4b. Ovarial gonomeres with central cavities; ×27.

FIG. 6. ZZ males treated with estradiol, preserved 4½ mo. after metamorphosis; a. completely feminized; b. with a testicular segment in the lower right gonad; × 5.

FIG. 7. Longitudinal section through lower right gonad of 6b showing hermaphrodite mosaic; × 40.

TABLE II. Double Treatment with Estradiol, 50 μg/l, Followed by Testosterone, 50 μg/l, until Metamorphosis.

	Treatment	♂	♀	♀
A.	Control	55	—	—
	Estradiol (7 days)	—	1	17
	Estradiol followed by testosterone	16	5	—
B.	Control	72	—	63
	Estradiol (7 days)	1	5	18
	Estradiol followed by testosterone	7	4	10

slightly modified in the following experiment. Genetic males (ZZ), treated at the proper time with estradiol, become changed into females, if reared later in pure water. However, if the estradiol treatment is immediately followed by administration of testosterone, the animals revert back to males. Table II presents 2 experiments with identical procedures. But in the first one (A) the mother was a sex reversed male (ZZ) while in the second (B) she was of normal ZW constitution. Both experiments show that estradiol treatments feminize offspring of the ZZ type; but if it is immediately followed by testosterone, the reversion is wiped out, all ZZ individuals become males again (or intersexes). Under exactly the same conditions the ZW individuals develop normally into females. This abolition of initiated sex reversal is the only indication that androgens may inhibit the cortical inductors in a similar though much less effective way as estrogens are suppressing the medullary system.

Conclusions and summary. Continuation of experiments on sex determination, particularly with Xenopus, led to the conclusion that the primary inductors, corticin (of follicle cells) and medullarin (of interstitial cells) are antigens. The corresponding *anti-corticin* and *anti-medullarin,* suppressing the contrary inductor system, are responsible for the unisexual (gonochoristic) development of each individual. Normally the decision of cortical (female) or medullary (male) prevalence depends on the quantitative relationship between sex determining genes. Experimental reversal of this influence does not produce, or depend on, any perceptible temporary or permanent change in gene composition or function. If administered at a short critical period of sex determination (St. 26/27), estradiol causes ZZ larvae to develop permanently as females. This effect is brought about by depression of the medullary inductor system. The possibly ensuing development of hermaphrodite glands indicates that selectively the capacity of antibody (anti-corticin) formation is more generally affected than that of the antigen (medullarin).

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Differential Distribution of Enzyme Hydrolyzing Acetylsalicylic Acid (Aspirin) in Certain Fluids of the Rat.* (22689)

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It appears likely that degradation of salicylic acid occurs principally, though probably not exclusively, in the kidneys since metabolites appearing in urine are not recoverable from the blood(1). The process is probably a complex one involving filtration at

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the glomerulus, tubular reabsorption, tubular conjugation and tubular excretion. However, the conversion of acetylsalicylic acid (hereinafter referred to simply as aspirin) into salicylic and acetic acids apparently takes place widely throughout the body(2), and it therefore becomes desirable to learn whether there is any differential distribution in various body fluids of the enzyme that catalyzes this hydrolysis. The object of the present study was to determine whether there are differences in rates of *in vitro* production of salicylic acid when aspirin is incubated in presumable immediate fluids of contact after gastrointestinal absorption in the rat (*i.e.*, abdominal thoracic duct lymph and portal vein blood) as contrasted with the systemically circulating blood.

Methods. Obtaining the fluids. Male Sprague-Dawley rats weighing between 175 and 225 g and full-fed on our usual adequate laboratory diet until the morning of the experiments, were used in 3 groups. In the group in which lymph was to be diverted for collection outside the body the animals were anesthetized with ether and the thoracic duct cannulated by the method of Bollman *et al.* (3). This technic comprises entrance into abdominal cavity through a large left flank incision, puncture of thoracic duct at approximately 1.5 cm above the receptaculum chyli, tying in a polyethylene tube (O.D. 0.062 cm, I.D. 0.045 cm) for drainage of lymph, and bringing the tube out of the cavity through puncture wound in midline of abdomen. On completing the silk-suture closure both the left flank wound site and that of the stab are painted over with collodion. The anesthetic is then withdrawn and lymph collection begins with animal confined in the Bollman cage(4), in which considerable freedom of movement is allowed without permitting claws or teeth to reach wound or protruding tube. Lymph was collected during 6 hours in a small centrifuge tube into which was placed at the beginning one small drop from a No. 27 needle of heparin sodium solution (10,000 units/ml) and a similar drop added as each ml of lymph accumulated. The collection tube was continuously twirled in beaker of ice water to insure that enzyme activity stopped at the moment a drop of lymph entered it and refrigerated at 4°C at end of sixth hour. In all instances, with 2 or 3 animals producing simultaneously, a lymph pool adequate for preparation of the aspirin solution was obtained through collections on 2 or 3 consecutive days. Animals bled from portal vein were anesthetized with ether, the abdomen opened in midline from manubrium sterni to symphysis pubis, the gastrointestinal structures displaced to gain access to the portal vein, the latter tied off close to point of entrance into the liver, and 2 ml of blood collected through syringe and needle, the latter containing 2 small drops of heparin solution, by direct puncture of the vein. The individual blood specimens were quickly cooled by twirling in beaker of ice water and a sufficient number accumulated to supply a plasma pool of equal size to that of lymph. The plasma obtained by centrifugation at 3500 r.p.m. for 10 minutes was stored in refrigerator. The blood specimens that were to represent systemically circulating blood were obtained by direct heart puncture under ether anesthesia of the third group of rats, cooling the 2 ml heparinized specimens as in the case of portal vein bloods and refrigerating the pooled plasma. *Determination of enzymatic hydrolysis.* On second or third day the 3 separate pools (lymph, portal vein plasma and heart's blood plasma) were filtered and 10 ml of each were placed in a separate flask into which aspirin had been previously introduced. The flasks were shaken vigorously for 2 minutes, then incubated at 38°C together with a flask containing only water and aspirin. The concentration of aspirin in all flasks was 50 mg/100 ml. At precisely 20, 40, 60, 80, 100 and 120 minutes after beginning of incubation, 1 cc aliquots were withdrawn from the flasks and the salicylic acid content determined by the method of Brodie *et al.*(5), in which the salicylic acid is extracted in acidulated ethylene dichloride, transferred to the water phase, an iron reagent added to give color, and read in the spectrophotometer at 540 millimicrons.

Results. The recoveries of salicylic acid from the 3 fluids are shown in Fig. 1, in which the data are charted as percentage recovered

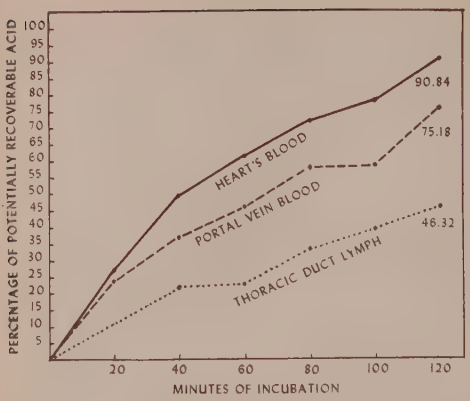

FIG. 1. Percentage recovered of potentially recoverable salicylic acid after *in vitro* incubation of acetylsalicylic acid in the respective fluids (rat).

of the potentially recoverable acid, using the means of 4 separate pools of each fluid at each of the time intervals. The highest recoveries at all intervals were from heart's blood, next highest from portal vein blood, and lowest from lymph. Throughout the entire 2-hour period the hydrolysis of aspirin proceeded at only about one-half the rate in lymph as in heart's blood. It is doubtful if the findings in the portal vein blood are truly representative of the enzymatic-hydrolysis-potential of that fluid directly as it leaves the site of principal aspirin absorption in the small intestine, because the specimens were taken without tying off the confluent veins from the stomach, spleen and pancreas, and therefore they contain some blood that had recently traversed the organs named. The presently designated portal vein blood is therefore actually such blood as it is delivered to the liver but it is not truly the first sanguine fluid that is encountered as aspirin is absorbed.

Discussion. The data establish that aspirin is hydrolyzed much less rapidly in lymph than in blood. It will be the objective of subsequent studies to determine whether the respective rates can be influenced by any adjuvant measures in the hope that the findings may help in the development of a satisfactory technic for determining salicylate antiphlogistic action in the laboratory.

Summary. The *in vitro* enzymatic hydrolysis of acetylsalicylic acid (aspirin) has been found to proceed only about one-half as fast in the abdominal thoracic duct lymph of the rat as in the heart's blood of the same animal.

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Two Types of γ-Globulin Differing in Carbohydrate Content. (22690)

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Considerable evidence has accumulated suggesting that certain antibodies in human (1,2,3) as well as horse sera(4,5) are of higher molecular weight than other antibodies and the bulk of γ-globulin. A sedimentation constant of approximately 19 S has usually been observed in the ultracentrifuge indicating a molecular weight close to 1 million instead of the ordinary 150,000. The possibility has been raised that these large proteins are aggregates of the low molecular weight components(6). Recent experiments employing zone electrophoresis(7) have furnished evidence that normal human γ-globulin contains from 5-10% of 19 S material as a constant constituent. This is localized primarily in the faster migrating portion of the γ-globulin. Gamma globulin prepared by

Deutsch employing alcohol fractionation has been found to contain significant amounts of this heavy component(1).

Preliminary observations(7) indicated that this high molecular weight γ-globulin was very rich in carbohydrate as compared to the main 7 S fraction. The latter was found to contain approximately 2.5% of carbohydrate consisting of hexose sugars, hexosamine and sialic acid which remained quite constant in various preparations. The present report describes further observations on the considerably higher carbohydrate content of the 19 S component of γ-globulin which has been purified by repeated preparative ultracentrifugation.

Methods. Zone electrophoresis was carried out primarily in a polyvinyl chloride supporting medium(7) because this was particularly suitable for carbohydrate analyses of isolated fractions. Starch was also used as supporting medium but not in those instances where carbohydrate analyses were done. Both media were employed in a similar manner and the amount of 19 S γ-globulin isolated by the two methods was quite comparable. Several preparations highly enriched in heavy material were prepared from pooled normal serum by preparative ultracentrifugation in a Spinco Model E ultracentrifuge. In one instance 864 cc of normal human serum were recycled 8 times at 114,000 x G. The times chosen were sufficient to sediment the heavy material into the bottom of the tube, and depended on the total protein concentrations of the solutions. Beginning with the fifth centrifugation albumin was added in an attempt to prevent denaturation of the heavy material. The final preparation, consisting of primarily 19 S material and albumin, was separated by zone electrophoresis employing polyvinyl chloride as a supporting medium. Analytical ultracentrifugation of the fractions was carried out in a Spinco Model E ultracentrifuge and the composition was determined by methods described by Trautman (8). Hexose sugars were determined colorimetrically using the anthrone method according to Mokrasch(9). The coefficient of variation (V) was 3.2%. The color obtained in the anthrone-glycoprotein reaction was corrected for the color caused by protein-sulfuric acid interaction. The O.D. of an equally treated protein-sulfuric acid blank was subtracted from the O.D. measured after the anthrone reaction. The hexose sugars present in serum glycoproteins which are responsible for the anthrone color are mannose, galactose and 6-deoxygalactose. The latter sugar was separately assayed by employing the cystine method described by Dische and Shettles(10). $V = 5.4\%$. For the determination of hexosamine the procedure described by Boas(11) was used chiefly. The proteins were hydrolyzed in 1.5 N HCl for 14 hours. Dowex 50 was used to eliminate non-specific chromogens and alkali treatment which is part of Ehrlich's reaction was carried out for 45 minutes at 90°C. $V = 3.8\%$.

Sialic acid was assayed according to the method of Werner and Odin(12) which is based on Bial's orcinol reaction. Although pentoses and hexuronic acid produce color in this reaction the absorption maxima of their color products differ greatly from the maximal absorption of the color caused by sialic acid. The absorption curves of 19 S γ-globulin and 19 S a_2-globulin after the orcinol reaction are almost parallel to that of sialic acid, and their absorption maxima coincide with that of the latter at 570 mμ. No other maximum was observed which could indicate the presence of either pentose or hexuronic acid. Crystalline sialic acid from ovarian cyst fluid was obtained through the kindness of Dr. Odin, Uppsala.

Results. Ultracentrifugal analysis of the various fractions of serum separated by zone electrophoresis in a starch or polyvinyl supporting medium has demonstrated that the 19 S component observed in whole serum is distributed in two electrophoretic fractions with peak concentrations in the γ_1 and a_2 regions(13). A similar distribution is obtained when the serum is first subjected to repeated cycling in the preparative ultracentrifuge and then separated by electrophoresis. Fig. 1 shows the ultracentrifuge pattern obtained after concentration of the heavy components in the preparative ultracentrifuge. The sample contained less than 15% material with a sedimentation constant below 19. All the

FIG. 1. Schlieren pattern obtained by ultracentrifuge analysis of a preparation of heavy component ($S_{20,w} = 19$ S).

albumin had been removed and only a small amount of 12 S and 7 S fractions remained. In this case no further albumin was added. The main peak after analysis at various concentrations was calculated to be $S_{20,w} = 19$ S. This sample was then subjected to zone electrophoresis in the starch medium and the protein pattern obtained is shown in Fig. 2. Two peaks are evident, one in the γ_1 and the other in the α_2 regions as indicated by comparison with the pattern of the original whole serum separated on the same starch block.

A number of different preparations of γ-globulin with varying amounts of heavy component were examined in the analytical ultracentrifuge and subjected to carbohydrate analysis. The amount of carbohydrate found was compared with the relative concentration of 19 S material. These results are plotted in Fig. 3. One of the major problems encountered in this work was progressive insolubility of the 19 S material after concentration and storage in the cold in the purified state. Associated with these alterations was the appearance of components with increased S

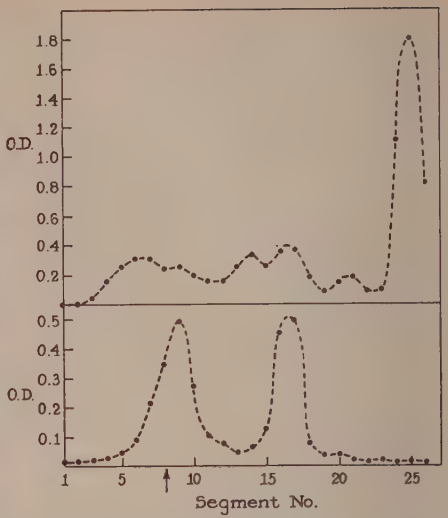

FIG. 2. Upper curve represents protein pattern obtained after electrophoretic separation of normal human serum. Lower curve represents a preparation of heavy component separated under identical conditions on the same starch block.

rates in the ultracentrifuge pattern. Since these materials were formed at the expense of the 19 S peak, they were included in Fig. 3 as belonging in the 19 S class. The question whether all of these heavier components arose in this manner was not entirely answered. Trace amounts were also found in some freshly isolated γ-globulin preparations. No evidence was obtained that the major 7 S fraction of γ-globulin aggregated in this manner to material with an S rate higher than 12.

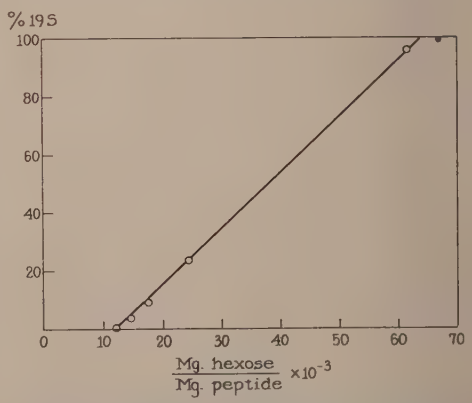

FIG. 3. Correlation between amount of 19 S material present in several γ-globulin preparations and the hexose-peptide ratio.

TABLE I. Carbohydrate Content of γ-Globulin and Various 19 S Proteins.

Protein	% N	% hexose	% fucose	% hexosamine	% sialic acid	% total carbohydrate	Hexosamine / Hexose
γ-glob. (7 S)	15.64	1.22	.29	1.14	.22	2.58	.94
γ_1 (19 S)	14.47	5.20	.62	2.90	1.70	9.80	.55
α_2 (19 S)	14.22	4.55		2.68	2.30	10.53	.59
Macroglob. I*	14.50	4.85	.62	2.66	1.71	9.22	.55

* Kindly furnished by Dr. H. F. Deutsch.

As shown in Fig. 3, there was a direct relationship between the percentage heavy components found in γ-globulin preparations and the carbohydrate-protein (peptide) ratio. The lowest point on the curve represents the ratio obtained for γ-globulin which is completely free of heavy components. The latter was obtained as previously described(7) from certain preparations of Fr. II-γ-globulin and from electrophoretic isolation of the slowly migrating material. The results obtained with 2 preparations which were close to 100% heavy component are indicated in the upper portion of the curve. The point, indicated as a solid dot, was not utilized in the plot because only a small amount of this preparation was available and the analyses were not considered to be as accurate as for the other points. Duplicate analyses were obtained for all the other preparations.

Table I shows the results of more detailed quantitation of the different carbohydrate components of one preparation which contained less than 5% of material with S rates lower than 19 S. For comparison the results obtained with 7 S γ-globulin and the α_2 heavy component are also indicated. It is apparent that the high molecular weight γ-globulin contains approximately 5 times as much hexose sugars as does the 7 S fraction. It has a low hexosamine-hexose ratio and is relatively rich in sialic acid. It is of interest that the α_2 fraction which is very similar to the γ_1 heavy component in size and shape(13) is also similar in carbohydrate content. Table I also gives the results obtained with a preparation of γ_1 material obtained from a case of Waldenström's macroglobulinemia. Immunological evidence obtained in this laboratory(14) and also by Korngold(15) has indicated a close relationship between the normal heavy component and the pathological macroglobulins. The carbohydrate content of this macroglobulin is similar to the normal preparation.

The sera of 4 patients (3 children and 1 adult) with agammaglobulinemia* were investigated to determine the concentration of the 19 S component migrating in the γ_1 region. When compared with normal sera a reduction to one-tenth or less of the normal level was found in 3 of the 4 cases. The methods used were not sensitive enough to indicate whether or not there was an even further reduction. Carbohydrate analyses on the fractions from one of these sera showed a complete absence of anthrone positive material throughout the γ_2 and γ_1 regions. The α_2 heavy component was not reduced in concentration in two of these sera in which it was examined.

Discussion. The high carbohydrate content of the 19 S group of proteins which migrate in the γ-globulin region demonstrates that these are not simple aggregates of the main 7 S type. They may represent polymerized molecules but if this is the case the basic units differ in hexose, hexosamine and sialic acid from other γ-globulins. Immunological studies which will be published separately (14) indicate that there are distinct antigenic differences between these 2 types of γ-globulin but that there is also considerable cross reaction. A similar immunological relationship was observed between 19 S and 7 S antibodies in horse sera by Treffers and associates(5). These observations suggest that the basic unit of the 19 S group of γ-globulins is similar to that of the 7 S class. The question of whether all the antigenic differences can be accounted for on the basis of the increased carbohydrate along with the large size of the heavy com-

* The authors are indebted to Dr. Robert Good for 3 of these sera.

ponents is not as yet answered.

It should be emphasized that the 19S γ globulin preparations analyzed in this study were never obtained 100% pure. Low molecular weight proteins could be almost completely removed and they presented a relatively minor problem. However the components with a sedimentation rate greater than 19S could never be eliminated entirely. The possibility remains that these materials may not be solely aggregates of the 19S fraction and cause slight alterations in the measured carbohydrate-protein ratio.

In the human, the blood group antibodies, certain abnormal red cell antibodies, and part of the Wasserman antibodies have been thought to be localized in the 19 S fraction of γ globulin. The possibility is raised that these and perhaps other antibodies are rich in carbohydrate. The increased carbohydrate, particularly sialic acid, would influence such properties as the isoelectric point and solubility and might confer unique characteristics to such antibodies.

The absence of detectable 19 S components of the γ_1-globulin type in the sera of 2 patients with agammaglobulinemia was reported previously(13). Further observations have confirmed these findings although small amounts were found in one case where some 7 S material was also present. These observations offer further evidence for a possible relationship between the heavy components under discussion and antibodies.

Summary. 1. The heavy components (19 S) have been highly concentrated from normal human serum by repeated cycling in the preparative ultracentrifuge. Electrophoresis of such preparations has permitted isolation of a high molecular weight γ-globulin fraction. 2. Analyses of this material indicate a high carbohydrate content with hexose sugars and sialic acid present at approximately 5 times the concentration found for the main 7 S γ-globulin. The hexosamine-hexose ratio is approximately 0.5. 3. Markedly reduced levels were found in the sera of 4 patients with agammaglobulinemia. The possibility that certain antibodies fall into the 19 S fraction and are similarly rich in carbohydrate is discussed.

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Oxidation of Acetate and Malonate in Extrahepatic Tissues.* (22691)

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Previous studies from this laboratory have shown that a rapid "explosive-like" oxidation of acetate occurs in the extrahepatic tissues (1). To investigate the pathway of terminal oxidation of acetate, the ability of C^{14} acetate to be oxidized in the presence of malonate, a competitive inhibitor of succinic dehydrogenase, was tested. This procedure, which has been successfully applied to *in vitro* studies (2,3) and which has been reported effective in whole animal experiments(4,5), proved to be non-inhibitory to acetate oxidation at concentrations of malonate that were not lethal to the eviscerated animals. Radioactive mal-

* This investigation was supported in part by research grant from the N.I.H., U.S.P.H.S.

TABLE I. Effect of Single Injection of Malonate on Oxidation of Radioactive Acetate. Results expressed as counts recovered in each period.

Period (30 min.)	Malonic acid administered 200 mg/kilo Exp. 511*	250 mg/kilo Exp. 474*
1	4364	3350
2	5391	3350
3	3293	2925
4	5031	2925
5	4563	3020
6	4648	2640
Avg	4550	3035
7	3219	3120
8	4421	2860
9	4632	2500
10	5263	2600
11	4519	2800
12	5249	3140
Avg	4560	2837

* In Exp. 511 the acetate was inj. at rate of 100 mg/kilo/hr calculated as acetic acid. In Exp. 474 tracer doses of acetate were administered.

onate was therefore utilized to investigate this apparent non-inhibitory effect of malonate on acetate oxidation. The results obtained are reported here.

Methods. General treatment of animals. Non-fasted rabbits were eviscerated-nephrectomized(6) and maintained at normal blood sugar at all times. A tracheal cannula was inserted at the time of operation to collect the expired CO_2 for C^{14} determinations. *Procedure used in determining the effect of malonate on acetate oxidation* (*Table I*). Carboxyl-labeled C^{14}-acetate was administered as the sodium salt and free acid (1:1) by constant intravenous infusion for 3 hours. At the start of the fourth hour sodium malonate† was administered intravenously as a single dose. The infusion of radioactive acetate was continued as before for another 3-hour period without interruption. By this procedure each animal acts as its own control for acetate oxidation. In the absence of excess carrier acetate the rate of acetate oxidation is constant, provided that the rate of acetate injection is constant. *Oxidation of malonate* (*Table II*). C^{14}-labeled sodium malonate was injected at

† Carboxyl labeled C^{14} disodium malonate was prepared from diethylmalonate purchased from Tracerlab, Inc. Methylene labelled C^{14} disodium malonate was prepared from dicalcium malonate purchased from Volk Radiochemical Co., Chicago, Ill.

TABLE II. Oxidation of C^{14} Labeled Malonic Acid after Single Intrav. Inj. Results expressed as mg/kilo/hr of malonic acid except in Exp. 476 where oxidation is expressed as % of inj. counts. Carboxyl labeled malonate was used in Exp. 476, 481, 478 and 480; and malonate-2-C^{14} was used in Exp. 509.

Hours	Exp. No. 476 Tracer	481 50	478 300	480 685	509 200
		Amt of malonic acid inj., mg/kilo			
1	5.0	3.0	7.9	18	2.7
2	8.5	3.4	13.4	17	3.3
3	6.5	2.8	13.5		2.9
4	5.9	1.9	8.1		3.5
5	3.7	1.5			3.7
Total % recovery	29.6	24	15		8
Total intracellular transfer of inj. dose (%)	58	55	60		

dosage levels (calculated as free acid) given in Table II. The expired CO_2 was collected each hour for C^{14} determinations. At the end of each experiment a plasma sample from each animal was also obtained for the C^{14} content. The total intracellular transfer of administered malonate was calculated from the counts administered and from the counts recovered in the terminal plasma samples. The extracellular space was assumed to be 22% of body weight. *Volume of distribution of injected malonate* (*Table III*). Plasma samples were taken at regular intervals after intravenous administration of radioactive malonate. These plasma samples were oxidized and the carbon collected and counted as $BaCO_3$.

Results. Effect of malonate administration on oxidation of acetate. It is apparent from the data in Table I that malonate ad-

TABLE III. Volume of Distribution of Injected Carboxyl Malonate. Results expressed as % of body wt (corrected for oxidation).

Time	Tracer Exp. 476	Amt of malonic acid inj., mg/kilo 50 Exp. 481	300 Exp. 478
20 min.	40	20	18
1 hr 20 min.	48	29	21
2 20		28	28
3 20	54	43	
4 20		33	37
5 20	65	34	

ministration in doses up to 250 mg/kilo does not inhibit oxidation of either tracer or carrier amounts of acetate.

Oxidation of malonate. The data in Table II show that both carboxyl and methylene C^{14}-labeled malonate are oxidized to CO_2 by the eviscerated animal. Rate of oxidation appears to be dependent on plasma concentration of malonate since the highest oxidation rate is found in those animals administered malonate at the higher dosage level. It should be pointed out that even in the animal with the highest malonate oxidation rate, the oxidation of malonate represents less than 2% of total metabolism of the animal. Intracellular transfer rate, which ranges from 55 to 60% over the 5-hour period of observation, suggests that cell entry is an important limiting factor in the rate of malonate oxidation. Dosages of malonate at 685 mg/kilo and higher proved to be quite toxic to the animal. This toxicity did not appear to be due to an inhibition of the citric acid cycle, but rather to the possible binding of metal ions by malonate. One indication of this was the lack of blood coagulation in the eviscerated animals treated with malonate.

Volume of distribution of injected malonate. The data in Table III show that injected malonate circulates initially only in a volume equivalent to the extracellular compartment. The intracellular transfer rate of malonate increases slowly with time. We consider the extracellular compartment to be about 20-25% of body weight.

Discussion. It has been shown (Table I) that under our conditions malonate does not have any apparent effect on oxidation of acetate to CO_2 by the eviscerated-nephrectomized rabbit. The lack of effect of even higher doses of malonate on acetate oxidation had been noted previously in the intact rat(7). The inability of malonate to inhibit acetate oxidation can be explained by 2 findings presented in this paper. First, volume of distribution studies show that cell permeability barriers exist for malonate, for approximately 40-50% of the injected dose is still circulating in the extracellular compartment 5 hours after malonate administration. Next, oxidation studies demonstrate that both carboxyl and methylene carbons of malonate can be oxidized. Thus, it can be seen that a slow rate of malonate entry into the cells coupled with its oxidation by the tissues, prevents intracellular concentration of malonate from attaining a level high enough to inhibit a succinic dehydrogenase.

The ability of the eviscerated animal to oxidize malonate is not surprising. Lifson and coworkers(8,9) studying C^{13} carboxyl labeled malonate conversion in the intact mouse and rat demonstrated the degradation of malonate to carbon dioxide. More recently Hayaishi(10) has reported that rat kidney homogenates were capable of producing isotopic CO_2 from radioactive malonate. Homogenates from rat liver and muscle, however, were found to be inactive. From our results it is clear that tissues other than kidney are also able to oxidize malonate.

A finding of particular interest to us is that a concentration of malonate much less than that normally used to demonstrate malonate inhibition in intact animals, was found to be lethal to the eviscerated animals. If one assumes that administration of this compound results in a chelation of metal ions, then it would appear that the *in vivo* administration of malonate could result in non-specific metabolic alterations which would allow very little experimental value for elucidating metabolic pathways. The finding of succinate(5) and glucose accumulation(4) by others after malonate injection could very well be a demonstration of malonate inhibition in the classical sense; however, this accumulation may also be due to causes other than a strict inhibition of succinic dehydrogenase.

Summary. 1. Oxidation of acetate to CO_2 by the eviscerated-nephrectomized rabbit is unaffected by malonate. 2. Studies on the fate of carbon-14 labeled malonate indicate that the intracellular transfer rate of malonate is low. 3. Conversion of radioactive malonate to CO_2 has been demonstrated in the eviscerated-nephrectomized rabbit. 4. The slow rate of cell entry and subsequent oxidation of malonate is offered as a possible explanation for the non-inhibitory effect of malonate on acetate oxidation.

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Cultivation of Type 2 Dengue Virus in Rhesus Kidney Tissue Culture.* (22692)

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It was reported(1) that mouse-adapted type 1 dengue virus of the Mochizuki and Hawaiian strains multiplied in trypsinized rhesus kidney tissue cultures, and produced a cytopathogenic effect. It was also demonstrated that degeneration of infected cells was inhibited by type specific anti-dengue immune rabbit serum, thus making possible virus neutralization tests in tissue culture. In the present paper, experiments are reported in which type 2 dengue virus was cultivated in tissue culture.

Materials and methods. Tissue cultures employed consisted of trypsinized rhesus kidney cells(2). Technical details including preparation and incubation of cultures, constituents of medium, etc., were described previously(1). Type 2 dengue virus of the New Guinea C strain was received on Jan. 25, 1955, from Dr. Edwin H. Lennette in the form of 20% mouse brain homogenate in 30% normal rabbit serum frozen with dry ice. It was stated that this material represented the 19th mouse brain passage, and that its LD_{50} titer was $10^{-6.2}$ at time of harvest, Nov. 11, 1953. The virus was kept in dry ice chest until used. On Feb. 1, 1955, the frozen material was thawed at room temperature, and diluted with Hanks' balanced salt solution. Its infectivity was measured by injecting 2 to 3-week-old white mice intracerebrally with 0.02 ml of 10-fold dilutions. The mortality ratios of injected mice were 4/4 for the 10^{-3} dilution and 0/4 each for dilutions of 10^{-4} through 10^{-8}.

Results. Serial passage through tissue culture: Seven cultures were inoculated with 0.2 ml of above-mentioned virus diluted 10^{-3}. After 10 minutes 1.8 ml of culture medium was added to each tube. The cultures were incubated in the stationary state at 35°C. Half or whole volume changes of medium were made usually every 2 to 3 days, to keep the pH of the fluid phase in the range of 7.2 to 7.6. Eleven days after beginning of incubation a portion of culture fluid was taken from each tube and pooled with fluids from other similar tubes. 0.2 ml of the mixture was then inoculated into new cultures with subsequent addition of 1.8 ml of medium to each tube. In this manner serial passages through tissue cultures were performed at intervals of 7 to 15 days. The virus content of the inoculum for each subculture was assayed by injecting 0.02 ml of 10-fold dilutions into mice. The results obtained are summarized in Table I. The specimens used for tissue culture passage and virus titration contained no agent cultivable on ordinary bacteriological culture media.

The virus was cultivated in this tissue culture system for 18 passages during 176 days.

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TABLE I. Serial Transmission of Type 2 Dengue Virus of New Guinea C Strain through Rhesus Kidney Tissue Culture.

Passage in tissue culture	Mortality ratios of mice inoculated with tissue culture fluid diluted 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Cumulative period of cultivation, days	Calculated dilution of original infected mouse brain based on passage and medium-change
1	2/3	1/3*	2/3	1/3	0/3	0/3	11	$10^{-4.9}$
2	3/3	2/3	0/3*	1/3	0/3	0/3	25	$10^{-6.8}$
3	0/3	0/3	0/3	0/3	0/3	0/3	38	$10^{-8.7}$
4	2/3	2/3	1/3	1/3	0/3	0/3	50	$10^{-12.0}$
5	1/3	0/3	2/3	1/3	0/3	0/3	65	$10^{-13.3}$
6	2/3	1/3	1/3	0/3	0/3	0/3	74	$10^{-15.2}$
7	0/3	1/3	1/3	0/3	0/3	0/3	81	$10^{-16.5}$
8	2/3	2/3	1/3	0/3	0/3	0/3	92	$10^{-18.1}$
9	1/3	0/3	2/3	0/3	0/3	0/3	100	$10^{-19.4}$
10	1/3	2/3*	0/3*	1/3	0/3	0/3	110	$10^{-21.3}$
18	3/3	2/3	0/3	2/3	1/3	0/3	176	$10^{-41.5}$†

* One of the surviving mice showed paralytic signs and recovered.
† For 1 ID_{50} in 18th passage.

One ID_{50} in the 18th passage represented a dilution of $10^{-41.5}$ of the original infected mouse brain homogenate. As reported by other investigators(3), mortality ratios of mice inoculated with the New Guinea C strain virus were erratic.

Morphological changes: Inoculated cultures were examined with ordinary light microscope. They showed cellular degeneration which was indistinguishable from that produced by type 1 dengue virus(1). Five days to one week after inoculation of virus, the cells were dark and spherical. This was followed by their detachment from the glass surface. After about 2 weeks only a relatively small part of the cell population remained. After 3 to 4 weeks practically all cells had disappeared. It was confirmed again in our experiments that uninoculated control cultures never showed widespread destruction of cells; changes resulting from prolonged incubation were limited. A large population of cells of normal appearance remained in uninfected cultures incubated 4 weeks or more.

Parallel titrations of virus in mice and in tissue cultures: The titers of cultivated virus were compared in mice and in tissue cultures. The inoculum was 0.02 ml for mice by the intracerebral route, and 0.2 ml for tissue cultures containing 1.8 ml of medium. The results obtained are given in Table II. In these limited tests the highest dilution which caused death of mice was greater than that producing definite degeneration of tissue culture cells.

Neutralization test in tissue culture: Immune serum was obtained by injecting a rabbit repeatedly with the New Guinea C strain of virus that had been propagated in mouse brain and had never been passed in tissue culture. The procedure for immunization was similar to that used in obtaining anti-type 1 immune serum(1). Serum collected from the rabbit prior to immunization served as a control non-immune serum. The serum was inactivated by heating at 56°C for 30 minutes, and stored at —10°C until used. Type 1 Mochizuki and Hawaiian strains of virus and

TABLE II. Parallel Titrations of Type 2 Dengue Virus of the New Guinea C Strain in Mice and in Tissue Cultures.

Passage in tissue culture	Inoc. into*	Dilution of infected tissue culture fluid 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10	Mice		1/3†	2/3	0/3	1/3	0/3	0/3
	Tissue cultures		3/3†	3/3	0/3	0/3	0/3	0/3
18	Mice	1/3	3/3	2/3	0/3	2/3	1/3	0/3
	Tissue cultures	3/3	3/3	3/3	2/3	0/3	0/3	0/3

* Inoculum: 0.02 ml in mice intracer., and 0.2 ml in tissue cultures with 1.8 ml of medium.
† Numerators indicate No. of mice showing characteristic signs of infection or tissue culture tubes showing definite degeneration. Denominators indicate No. of mice or tissue culture tubes inoculated.

TABLE III. Neutralization Tests in Tissue Cultures of Type 1 and Type 2 Dengue Viruses.

Virus					Serum*		
Type	Strain	Passage in tissue culture	ID_{50} for tissue culture/0.2 ml	Dilution of infected tissue culture fluid	Anti-Mochizuki immune	Anti-New Guinea C immune	Control†
1	Mochizuki	43	$10^{-5.0}$	100-fold	0/5‡	5/5	5/5
	Hawaiian	25	$10^{-5.25}$	"	0/5	5/5	5/5
2	New Guinea C	18	$10^{-3.25}$		5/5§	0/5	5/5

* Diluted 1:5 with 0.1% lactalbumin hydrolysate medium. Aliquots of this mixed with equal volumes of virus suspension.

† Taken before immunization from a rabbit used for immunization against New Guinea C strain virus.

‡ Numerators indicate No. of tissue culture tubes showing definite degeneration. Denominators indicate No. of tissue culture tubes inoculated. The culture fluid used for changing medium included 1% of immune or control serum.

§ Degeneration of tissue cultures inoculated with mixture of type 2 virus and anti-type 1 serum was milder than that in control cultures exposed to type 2 virus and non-immune serum.

anti-Mochizuki immune rabbit serum were used to determine heterologous reactions. In performing the neutralization tests a given concentration of cultivated virus was mixed in equal volume with serum diluted 1:5 in 0.1% lactalbumin hydrolysate medium. After incubation at 37°C for 1 hr and at 4°C for 1 more hr, 0.2 ml of the mixture was inoculated into tissue culture tubes with subsequent addition of 1.8 ml of medium. Examples of results obtained are shown in Table III.

It was demonstrated that the viruses were neutralized by the homologous serum, but not by the heterologous serum. It appeared, however, that the degeneration shown in tissue cultures inoculated with the mixture of type 2 virus and anti-type 1 serum was a little milder than that shown in control cultures exposed to virus and non-immune serum.

Summary and conclusions. 1) Mouse-passaged type 2 dengue virus of the New Guinea C strain was cultivated in trypsinized rhesus kidney tissue cultures grown directly on glass surfaces and incubated in the stationary state at 35°C. The virus was maintained in this system of tissue culture through 18 passages during 176 days. Culture fluid from the 18th passage representing a $10^{-41.5}$ dilution of the original infected mouse brain was infective for mice. 2) The infected culture cells exhibited characteristic degeneration indistinguishable from that associated with infection of tissue cultures with type 1 dengue virus. Infectivity of the virus was neutralized by specific antiserum as indicated by the fact that the degeneration was suppressed completely by homologous immune rabbit serum, but not by heterologous serum. It appeared that the cellular degeneration produced by type 2 virus mixed with anti-type 1 serum was a little milder than that produced by the same virus mixed with the control non-immune serum. Although further studies are necessary to clarify the antigenic relationships of type 1 and type 2 dengue viruses, it is possible that the reduced degeneration was due to a partial neutralization by heterologous antibody.

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Anaerobic Loss of Endogenous Glycogen in Rat Heart Slices. (22693)

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In the course of a series of comparative studies it was found that rapid depletion of the endogenous glycogen of rat heart occurred *in vitro* under anaerobic conditions. Attempts to demonstrate an accumulation of lactic acid under these conditions were unsuccessful and lactic acid was actually found to disappear slowly during anaerobic incubations. A summary of typical data appear in Table I. No significant concentration of preformed or 7 minute hydrolyzable reducing substances, pyruvate, acetoin, citrate, volatile acids or triose phosphates was found in any time period. Small concentrations of fructose and high concentrations of hexosemonophosphate were demonstrated in all time periods. Very high concentrations of 5 hour hydrolyzable reducing substances of undetermined origin were found in all time periods but wide variations between individual experiments were also found: 898 μg/100 mg (542-1131) at zero time, 288 (107-392) at 10 minutes, and 760 (733-851) at 30 minutes. On the basis of these results the following compounds are thought to be excluded as end products of the observed glycogen breakdown in heart slices: glucose, hexose phosphates and diphosphate, fructose, glyceraldehyde phosphate and dihydroxyacetone phosphate, pyruvate and lactate, acetoin, acetate and citrate.

An attempt was made to evaluate this unusual finding in the whole animal. To simulate the anaerobic *in vitro* incubations the heart was allowed to remain within the chest for varying times after the rat was beheaded. Under these conditions the endogenous glycogen of the heart fell rapidly but there was a concurrent accumulation of lactic acid, in agreement with the usual glycolytic theory (Table II).

The "extra" lactate formed in this system probably represents that formed in the terminal metabolism from the various glycolytic intermediates.

Discussion. A survey of the literature failed to reveal a comparable study. In a series of reports in 1932-35, Haarman indicated that glycogen added to heart *brei* was destroyed but that lactic acid was not formed (15,16). From our experiences with attempting to prepare a heart brei it seems that Haarman's work should be considered a study of a variety of isolated soluble enzyme systems from heart. Barron, *et al.*(17) have shown that anaerobic glycolysis in rat heart slices which have been thoroughly oxygenated before incubation leads to an accumulation of lactic acid as indicated by chemical analysis and CO_2 production from the buffer. This apparent contradiction of our results is probably the result of different pre-treatments of the slices. In the present study the slices were collected in nitrogen-CO_2-gassed buffer rather than in oxygen-gassed buffer. The demonstration that hexosemonophosphate accumulates in the skeletal muscle of rats killed by decapitation or similar means(18), led us to believe that hexosemonophosphate was the accumulated end product of the observed glycogen breakdown. Determination of the hexosemonophosphate content of the heart slices at the various time periods showed a high concentration but no accumulation of this intermediate. The possibility that the failure of lactic acid to accumulate may reflect the operation of an alternate pathway of carbohydrate metabolism, such as those known for hexose(19) and pyruvate(20), has not been investigated. The report(17) that lactic acid accumulates as expected when the tissue has been thoroughly oxygenated or, as here, when incubation of the tissue takes place *in situ*, leaves some question as to the physiological significance of such a pathway.

Experimental. Five- to six-month-old Supplee white rats of both sexes were used. All preparations were carried out at 0-3° unless otherwise indicated.

In vitro incubations: The rats were beheaded and the heart removed immediately

TABLE I. Disappearance of Glycogen and Lactic Acid *In Vitro.*

Sample	Elapsed time, min.	Glycogen, μg/100 mg*	Lactic acid, μg/100 mg	CO_2, μl/flask
Pre-equilibration	0	273 (8)	75 (8)	
Post-equilibration	10	138 (4)	57 (4)	
Post-incubation†	40	20 (4)	46 (4)	0 (24)

* Wet wt.

† Incubations at 25° or 38°. Data typical of either temperature. Numbers in parentheses are No. of separate analyses or observations.

to an appropriately gassed buffer solution (pH 7.4 Krebs Ringer bicarbonate or phosphate (for acetate study) buffers(9)). After about 10 seconds the heart was removed, the auricles and major vessels trimmed off, and 0.5 mm slices prepared with a Stadie slicer (10). The first slice was discarded and the others placed in a fresh gassed buffer solution. The slices were blotted dry on filter paper and 100-300 mg (depending on the experiment) transferred to 3 ml of gassed buffer in standard Warburg vessels. The vessels were equilibrated at 25°C or 38°C under flowing nitrogen-CO_2 (95%-5%) or nitrogen (acetate study) for 10 minutes and then incubated 30 minutes by the standard Warburg technic. The reaction was stopped by the addition of 0.3 ml 35% trichloroacetic acid (TCA) or 0.3 ml 24 *N* H_2SO_4 (acetate study). The flask contents were then homogenized, centrifuged (except acetate samples) and the supernatant analyzed.

In situ incubations: Rats were beheaded and the heart removed from the chest at the indicated time to gassed buffer solution. The washed heart was cut in half and one-half homogenized in a fresh buffer solution containing TCA. Lactate analyses were performed on the supernatant while glycogen was determined on the remaining half heart.

The following analytical methods were employed: glycogen(11), lactate(12), glucose and reducing substances(13,14,15), fructose (16), hexosemonophosphate(17), pyruvate (18), acetate(19), acetoin(20), triosephosphates(21) and citrate(22).

Summary. An anomalous situation has been encountered in a study of the endogenous carbohydrate metabolism of heart. It is believed that this anomaly, the failure of lactic acid to accumulate during anaerobic glycogen breakdown, may reflect the operation of an alternate pathway.

TABLE II. Changes in Glycogen and Lactic Acid *In Situ.*

Time after beheading, min.	Glycogen, μg/100 mg	Lactic acid, μg/100 mg	% recovery (glycogen as lactate)
0	249	84	
2	196	175	
5	196	184	
10	46	306	
30	39	335	119.8%

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Effect of N-Allylnormorphine Upon Massive Doses of Narcotic Drugs. (22694)

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Although N-allylnormorphine (nalorphine) is an effective antagonist against a wide spectrum of pharmacological actions of opiates (1,2), and has proven to be clinically useful in cases of narcotic overdosage(3-6), Koppanyi and Karczmar(7) did not succeed in saving mice from lethal doses of morphine by single injections of nalorphine. On the other hand, Unna(8) reported that nalorphine in divided doses reduced mortality in mice receiving 800 mg/kg of morphine, and Gruber (9) showed that even a single dose of the antagonist was protective against morphine sulfate up to 800 mg/kg if the ratio of the two drugs was properly chosen.

The present experiments demonstrate a difference in the protective effect of nalorphine against lethal doses of various opiates and a species difference in the responses.

Experimental. Preliminary experiments were performed in rabbits receiving meperidine hydrochloride by slow intravenous injection. The dose of meperidine used was 25 mg/kg, except in two rabbits in which severe convulsions forced discontinuance of the injection at 15 and 20 mg/kg, respectively. The results (Groups I-III, Table I) show that nalorphine, administered subcutaneously 30 minutes before the opiate, was in no case successful in preventing convulsions. In each case where death occurred, respirations ceased at about the same time convulsions set in. Respirations were maintained, however, in the meperidine-injected rabbits pre-treated with 10 mg/kg of nalorphine (Group III). In these, the convulsant action of the opiate persisted for 4 to 10 minutes, and the animals then recovered. Two animals (Group IV) receiving the anticonvulsant agent, phenobarbital, in addition to nalorphine exhibited neither convulsions nor grossly visible respiratory depression when 25 mg/kg of meperidine was injected.

These preliminary observations in rabbits suggested that nalorphine antagonized the respiratory depressant effect of the opiate without markedly influencing the convulsant activity. In Group III, sufficient nalorphine was administered so that respiration did not stop and the animals survived, but the convulsions were as severe as in the previous groups. It appeared, then, that convulsant activity might be one effect of opiates that nalorphine would not antagonize. In order to test this further, experiments were conducted in mice and rats.

For these experiments, male IS32 mice of about 20 g body weight and male Holtzman rats of about 130 g were employed. Table II

TABLE I. Effect of N-Allylnormorphine upon Convulsant and Lethal Effects of Intravenous Meperidine in Rabbits.

Group	Antidote	No. of animals	No. convulsing	No. surviving
I	None	2	2	0
II	Nalorphine, 1 mg/kg	2	2	0
III	Nalorphine, 10 mg/kg	4	4	4
IV	Nalorphine, 10 mg /kg + phenobarbital, 100 mg/kg	2	0	2

TABLE II. Protection of Mice by Nalorphine Hydrochloride against Lethal Doses of Morphine or Methadone.

Morphine sulfate			Methadone hydrochloride		
	No. dead/total			No. dead/total	
Dose, mg/kg	Without nalorphine	With nalorphine*	Dose, mg/kg	Without nalorphine	With nalorphine†
350	6/10	0/10	25	3/10	0/10
440	7/10	2/10	29.5	7/10	0/10
555	7/10	1/10	35	8/10	0/10
700	10/10	5/10	41	8/10	0/10
			50	9/10	0/10
			70	10/10	2/10

* Nalorphine HCl dose: 25 mg/kg, repeated at ½- to 1-hr intervals until total of 225 mg/kg had been given.

† Nalorphine HCl dose: 10 mg/kg 15 min. before methadone, repeated 1 hr and 6 hr after methadone.

demonstrates that, contrary to the findings of Koppanyi and Karczmar (*loc. cit.*), adequate doses of nalorphine offer considerable protection to mice receiving lethal doses of morphine. It is evident, however, that nalorphine is more effective against methadone than against morphine in mice. One difference was observed between the mice receiving morphine and those receiving methadone: These high doses of morphine produced convulsions in the animals, while methadone did not. The results are therefore consistent with the finding in rabbits that nalorphine is less effective against convulsions than against respiratory depression.

Codeine phosphate and prisilidene hydrochloride are convulsant drugs at toxic dose levels in mice, but not in rats. In the latter species, hypnosis, catalepsy and respiratory depression are the prominent effects of toxic doses of these drugs, and convulsions do not develop except terminally (probably asphyxial in origin). In Table III, it is shown that rats, but not mice, are easily protected by nalorphine against lethal doses of these drugs.

Another experiment therefore was performed in which an anticonvulsant drug was included. Forty mice were divided into 4

TABLE III. Species Difference in Response of Rats and Mice to Nalorphine Hydrochloride when Given Lethal Doses of Codeine or Prisilidene.

Mice			Rats		
	No. dead/total			No. dead/total	
Dose, mg/kg	Without nalorphine	With nalorphine*	Dose, mg/kg	Without nalorphine	With nalorphine†
		A. Codeine phosphate			
300	3/10	3/10	500	7/10	0/10
330	3/10	4/10	700	5/ 5	0/ 5
363	2/10	9/10	1000	5/ 5	0/ 5
400	9/10	10/10			

* Nalorphine HCl dose: 25 mg/kg immediately before codeine, repeated ½ hr later. Death occurred before a third dose could be given.

† Nalorphine HCl dose: 25 mg/kg ½ hr before codeine, repeated 1 hr and 4 hr after codeine.

B. Prisilidene hydrochloride

Mice			Rats		
50	0/10	0/10*	40	5/5	0/5†
72	1/10	2/10	60	4/5	0/5
104	7/10	5/10	80	5/5	0/5
150	9/10	10/10			

* Nalorphine HCl dose: 25 mg/kg repeated at ½-hr intervals to a total of 200 mg/kg.

† Nalorphine HCl dose: 25 mg/kg—no repetition.

TABLE IV. Effect of Combination of Anticonvulsant Drug and Nalorphine in Mice Receiving Lethal Dose of Codeine.

Group	No. of animals	Pretreatment	Effect of codeine PO_4 (450 mg/kg) No. animals Convulsing	Dead
I	10	Phenobarbital Na, 50 mg/kg	2	9
II	10	Nalorphine HCl, 50 mg/kg	10	8
III	10	Combination	1	1
IV	10	None	10	10

groups, with drugs injected subcutaneously as follows: *I*. Phenobarbital sodium 50 mg/kg. *II*. Nalorphine hydrochloride 50 mg/kg. *III*. Both phenobarbital and nalorphine, each 50 mg/kg. *IV*. Uninjected controls. One-half hour later, the animals received codeine phosphate 450 mg/kg, a dose slightly above the LD_{100} according to the data in Table III. The results of this experiment are shown in Table IV. It is apparent that phenobarbital alone controlled the convulsions in most of the mice, but did not prevent death. Nalorphine alone failed to control the convulsions, and saved the life of only 2 of 10 mice. When both nalorphine and phenobarbital were given, convulsions and death were prevented in 9 of 10 animals.

Discussion. It appears from these experiments that not all of the effects of opiates are equally well inhibited by nalorphine. One effect in particular seems to be poorly antagonized, and that is the convulsant action. The tendency of narcotic analgesic drugs to produce convulsions varies among drugs and among species. For example, large doses of morphine are convulsant in some species, but convulsions are rare in man(10). Our results indicate that codeine and prisilidene are highly convulsant for mice, but not for rats. If one assumes that nalorphine competes with morphine-like drugs at their sites of action, it is possible that it has less affinity for sites responsible for convulsions than do the convulsant opiates.

Summary. Nalorphine is an effective antidote for doses of opiates which would otherwise be lethal, in those instances where signs of depression are the dominant toxic manifestations. It is relatively ineffective, however, in those instances where convulsions are a prominent feature. In rabbits and mice receiving convulsant doses of meperidine, codeine or prisilidene, nalorphine alone was seldom life-saving, but administration of both phenobarbital and nalorphine adequately protected the animals. Codeine and prisilidene did not produce convulsions in rats; nalorphine alone was an effective antidote in these instances.

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Cyclic Variation in the Mule Deer Thymus.* (22695)

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A series of fawn, yearling, and adult female mule deer (*Odocoileus hemionus hemionus*) was collected on the National Bison Range, Moiese, Mont. for purposes of studying the reproductive cycle of the female mule deer. Specimens of each of the 3 age groups were killed each month between Aug., 1953 and Sept., 1954. Measurements, tissue weights, and tissue preservation were made in the field. One of the tissues routinely measured, and portions preserved, was the bilobed thymus gland.

Method. The female deer were hunted, shot, and autopsied on the open range. All measurements were made on the fresh thymus *in situ,* each lobe being measured separately. The length, width at the middle, and the thickness in the center transections were taken with a celluloid rule. A portion of each gland was removed and preserved in Bouin's fixative. No histological study of this material has been made to date. The volume of each gland was calculated by assuming the gland to be an elliptical spheroid. The calculated volumes of the 2 lobes were averaged and divided by the total body weight of the animal to obtain the thymus volume/body weight ratios used in Fig. 1. The curve of the thymus volume/body weight represents the combined average values of the three age classes for each month. The total number of animals used was: 22 fawns, 22 yearlings, and 26 adult female mule deer.

Results. All 3 classes exhibited the same type of annual thymus volume cycle, although the fawns had the highest value of the 3 age classes for the late spring-early summer months. The pregnant adult does had the least high value of the 3 age classes for that time of year, and the yearlings had intermediate values. All 3 age classes were equally low during the winter months whether they were pregnant or non-pregnant.

The thymus gland is commonly used as an indicator of systemic stress(1), but it seemed conjectural to us whether or not in this case the thymus gland could be used as an indicator of systemic stress. The annual variation in thymus gland volume might well represent stress factors of high temperatures and "burnt up" vegetation in the summer months, and cold temperatures and difficulty of obtaining feed in the snowy winter months. The slower recovery of thymus volumes in the yearlings and adults in the early spring can be attributed to placental hormones from December to June. Non-pregnant fawns had thymi with values as low as the pregnant yearlings and adults during December, January, and February.

The data also permit the hypothesis that light stimulates the production of a thymotropic hormone via the pituitary gland. This hypothesis seems as reasonable as that of stress when the close correlation is noted between the average monthly hours of daylight and the thymus volume/body weight curves, (Fig. 1). Also there was no indication of annual adrenal cycle in weight, volume, or cortex-medulla ratio which is believed to be intimately involved in any symptoms of systemic stress.

The apparent relationship of the thymus volume in the mule deer to climatic conditions revealed in this study warrant the attention of other investigators. This is particularly appropriate since circumstances prevent the present authors from immediately following up the ramifications of the problem on either domestic, laboratory, or game animals.

Summary. Thymus glands from a series of fawn, yearling, and adult female mule deer, collected on the National Bison Range, Moiese, Mont., varied in volume with the season. The thymus reached its lowest volume during the winter months (Dec.-Feb.)

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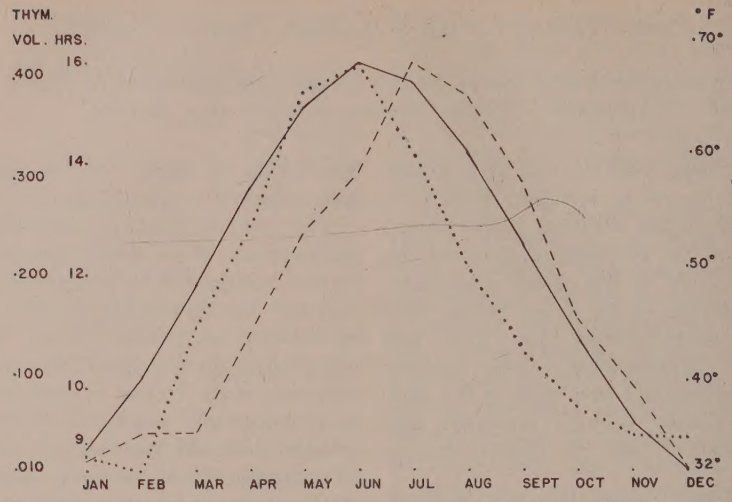

FIG. 1. Annual weather cycle, and thymus volume of female mule deer, National Bison Range, 1953, 1954. Avg monthly day length, ———; avg monthly temperature, 1953-1954, – – – –; avg monthly thymus vol/body wt ratios of female mule deer of all age groups, • • • • •

and reached its largest size in all 3 age classes during late spring and early summer (May-July). All 3 groups exhibited the same type of annual thymus volume cycle, although the fawns had the highest value of the 3 age classes for the late spring-early summer months. Pregnant or lactating adult does had the lowest value of the 3 age classes for that time of year, although these values were considerably higher than those of the winter months. The data permit the hypothesis that light stimulates production of a thymotropic hormone probably via the pituitary gland. There is very good correlation between average monthly hours of daylight and thymus volume/body weight curves. Stress factors such as high temperatures and "burnt up" vegetation in late summer, and cold temperatures and difficultly available food in snowy winter months may also affect the size of the thymus.

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Obtaining Data by Telephone. A Clinical Evaluation of Hypnotic Drugs. (22696)

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The widespread use of hypnotic drugs suggests that many people require some aid to fall asleep. Repeated studies at the Indianapolis General Hospital using the usual doses of various hypnotics have failed not only to demonstrate differences among hypnotic agents but also between the hypnotics and placebo. This paper presents our experience with telephone reporting.

Method. Tablets of Ethinamate (Valmid), Glutethimide (Doriden), Methyprylon (Noludar), and secobarbital sodium (Seconal Sodium) containing 500 mg, 500 mg, 200 mg, and 100 mg, respectively, were used. Envelopes containing one or 1½ tablets of these hypnotics or placebo were given to patients

TABLE I. Reported Time Lapse Prior to Onset of Sleep. Condensed form of data used in analysis. (Numbers in body of table refer to No. of reports in each category.)

Onset in min.	Tablets Placebo		Tablets Seconal		Tablets Valmid		Tablets Doriden		Tablets Noludar	
	I	II	I	II	I	II	I	II	I	II
0- 30	9	13	17	18	13	15	13	14	16	9
31- 60	9	4	6	5	5	6	7	12	7	7
61-120	5	7	3	2	9	6	4	1	2	6
121-480	4	3	1	2	0	0	3	0	2	5

receiving out-patient care. These envelopes were coded and directions as to order of administration given to the patients. The order of administration was also noted on the report form. The medications were given in a planned order so that randomization was obtained. Each morning the patients called the hospital to report the results of the previous night. Any of several persons answered the telephone and recorded the data. The questions were: The envelope number, the number of tablets taken, how soon they fell asleep, how long they slept, how well they rested, whether they were dizzy or drowsy when they awoke, whether they liked the medications and whether it helped them to sleep. Any other statements relating to the medication were also recorded. After the first 2 or 3 calls, some of the patients discontinued calling. They were called the same day to obtain their report and learn their reason for not calling. Following this show of interest on the part of the investigators, most of the patients did not fail again. Despite the many diversions which occurred, it was thought that adequate and accurate records had been obtained from 16 patients. These 16 patients completed the series, some several times, for a total of 27 complete reports on each medication at each dose.

Results. In the analysis of the data equal emphasis was placed on each of the 27 completed series of treatments. Since each series included the administration of each of 5 drugs at 2 doses, 270 daily reports were obtained. In plotting the curve for the onset of sleep (Table I) a skew distribution was observed. Transformation to logarithms, therefore, preceded variance analysis. Variance analysis indicates that sleep was significantly more prompt when hypnotics were used than when placebo was given. The differences among the drugs and between the 2 doses were not significant.

Analysis of the data on the duration of sleep (Table II) indicates significant heterogeneity among the hypnotics but placebo was not significantly different from the over-all hypnotic effect. This was an unexpected effect which on a clinical basis was illogical. Therefore, any comparison of the duration of sleep was not considered valid.

Analysis as to the number of times awakened, side-effects, and other data demonstrated no significant differences among the hypnotics, between the hypnotics and placebo, or between the two doses of drugs(1).

Discussion. By using adequate doses, differences among hypnotic drugs can be demonstrated in animals(2) and men(3,4). In these studies both the onset and duration of the central depression was measured. It was assumed that patients who had difficulty getting to sleep were capable of recognizing the effects of any drugs which enabled them to achieve the sleeping state more promptly.

TABLE II. Reported Durations of Sleep. Condensed form of data used in the analysis. (Numbers in body of table refer to No. of reports in each category.)

Duration in hr	Tablets Placebo		Tablets Seconal		Tablets Valmid		Tablets Doriden		Tablets Noludar	
	I	II	I	II	I	II	I	II	I	II
0 - 4	5	2	2	3	2	2	3	1	5	6
4.5- 6	10	5	5	4	11	8	4	7	2	8
6.5- 8	9	15	17	14	12	14	17	14	17	10
8.5-12	3	5	3	6	2	3	3	5	3	3

Patients were able to recognize a more rapid onset of sleep with hypnotics than with placebo. Unless the hypnotics were long acting and produced a "hangover" no after effects would be expected and none were noted.

Conclusions. 1. The onset of sleep is more prompt when usual therapeutic doses of hypnotics are administered to patients. 2. Duration of sleep and incidence of undesirable effects produced by Seconal, Valmid, Doriden, and Noludar were not significantly different from those produced by placebo.

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